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PATENT
12839/1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s)	:	Alexandre MARTI et al.
Serial No.	:	09/673,817
Filed	:	April 22, 1999
Title	:	SOLUTION FOR DIAGNOSING OR TREATING TISSUE PATHOLOGIES
Art Unit	:	1617
Examiner	:	S. J. Sharareh

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. § 1.132

SIR:

I, GEORGES WAGNIÈRES, Ph. D., declare as follows:

1. I am a joint inventor of the subject matter of the above-identified application.

2. I received a doctorate degree from the Swiss Federal Institute of Technology (EPFL) in 1992. From July 1993 to August 1994, I was a postdoctoral fellow at the Harvard Medical School, Wellman Laboratories of Photomedicine, Boston Massachusetts.

3. I am currently an Adjoint scientifique (Project Leader) at the Swiss Federal Institute of Technology (EPFL) in Lausanne, Switzerland, and have served in this capacity since September 1994. From May 1992 to June 1993, I also served in the same capacity as I currently do at the EPFL.

4. In the past fourteen years, I have been a co-author of at least 69 papers published in peer-reviewed journals and have been an author or coauthor of 6 books, as well as over 60 other publications. Many of these publications are in the area of photodynamic therapy and diagnosis. (*See, e.g.*, F. Ludicke et al. "Photodynamic diagnosis of ovarian cancer using aminolevulinic acid hexylester: A preclinical study", Brit. J. of Cancer, 88(11), pp 1780-

1784, 2003; Radu A. et al. "Photodynamic therapy of early squamous cell cancers of the esophagus", Gastrointestinal Endoscopy Clin. North America, 10(3), pp 439-460, 2000).

5. I am a member of a number of professional societies, including the International Society for Optical Engineering, the Optical Society of America and the European Optical Society. I have served on the editorial board of a journal specializing in diagnostic optics, The Journal of Biomedical Optics, and have chaired numerous international conferences. Additional facts about my background and qualifications including a list of my publications are set forth in my *curriculum vitae*, attached as **Exhibit A**. An updated list of published articles is attached at the back of my *curriculum vitae*.

6. I have read and understand the outstanding Office Action mailed July 25, 2003 and the cited U.S. Patent No. 6,034,267 (hereinafter the "'267 patent"), issued on March 7, 2000 to Gierskcky et al.

7. The present application provides a solution for administration to a patient for diagnosis or treatment comprising a physiologically acceptable solvent and an ester of 5-aminolevulinic acid (E-ALA) which is present in the solution at a concentration of less than 1% by weight.

8. My co-inventors and I, and/or those working under our direction and supervision, performed experiments described in the application and obtained the results discussed therein. Below I provide additional details on these results, as well as subsequent studies.

9. Protoporphyrin IX ("PpIX"), a heme precursor, is used as a fluorescence marker and photosensitizing agent in photodynamic therapy. PpIX forms and accumulates in tissues with a high cellular turnover, *e.g.*, tumors. Therefore, an increase in intracellularly generated PpIX formation in response to exogenous stimulation by administration of 5-aminolevulinic acid (ALA) may be used for tumor destruction by photodynamic (PDT) therapy. ALA-mediated photodynamic therapy (PDT) was an emerging field for treatment of cancers since about 1989-1990. However, since ALA-mediated PDT is limited by ALA's poor ability to diffuse through cell membranes, solutions containing high doses of ALA of about 180 mM (about 3% w/w) have to be administered to increase PpIX production in the deep layers of cancerous lesions. Therefore, I have studied the comparative effects of administration ALA-esters and of ALA to determine optimal concentrations of these PpIX precursors for use in PDT and photodiagnosis.

10. In one study, two cell lines derived from human transitional cell carcinoma of the bladder, J82 and T24 cells, respectively, were incubated with ALA or esters of ALA, as described in P. Uehlinger et al. "5-Aminolevulinic acid and its derivatives: physical chemical properties and protoporphyrin IX formation in cultured cells", J. Photochem. Photobiol. B: Biol., 54, pp 72-80, 2000 (**Exhibit B**). The esters of ALA studied included ALA-methylester, ALA-ethylester, ALA-butylester, ALA-hexylester, and ALA-octylester.

11. In this study, I found that ALA-butylester, ALA-hexylester, and ALA-octylester not only produced PpIX formation at much lower concentrations than ALA, but also produced higher amounts of PpIX, as shown in the graphs for the J82 and T24 cells, respectively, which are adapted from Figure 3 of Uehlinger et al. 2000, and are attached as **Exhibit C**.

12. Experiments were also performed on bladder tissue *in vitro*, in which PpIX formation after administration of ALA or ALA-ethylester, ALA-butylester, ALA-hexylester, and ALA-octylester, were measured, as described in A. Marti et al. "Optimization of the Formation and Distribution of Protoporphyrin IX in the Urothelium: an In Vitro Approach", J. Urology, 162(2), pp 546-555, 1999, attached as **Exhibit D**. The results again demonstrated higher amounts of PpIX formation at much lower concentrations of ALA-ethylester, ALA-butylester, ALA-hexylester, and ALA-octylester than with ALA. These results are shown in **Exhibit E**, adapted from Figure 4 of Marti et al. 1999. A Table summarizing these results, attached as **Exhibit F**, shows the optimal concentrations of ALA and ALA esters for PpIX production. The Table (**Exhibit F**) indicates that the ALA-butylester, ALA-hexylester and ALA-octylester all achieved optimal PpIX levels at concentrations of less than 1%. The data in **Exhibits E** and **F** demonstrate that various concentrations below 1% of ALA-ethylester, ALA-butylester, ALA-hexylester, and ALA-octylester achieve high levels of PpIX formation.

13. *In vivo* clinical studies were performed to compare induction of PpIX with 8 mM of ALA-hexylester and 180 mM of ALA in a human pTa G2 cancer patient, as described in Lange et al. "Photodetection of early human bladder cancer based on the fluorescence of 5-aminolaevulinic acid hexylester-induced protoporphyrin IX: a pilot study, Br. J. Cancer, 80(1/2), pp 185-193, 1999 (**Exhibit G**). For ALA-HCl, 180 mM corresponds to 30.17 mg/ml or 3.02% (w/w), whereas 8 mM of ALA-hexylester HCl corresponds to 2 mg/ml or 0.2% (w/w). The results, derived from Lange et al, p. 190, last paragraph, are shown in **Exhibit H**. They demonstrate that higher levels of PpIX were formed with much lower concentrations of ALA-hexylester than ALA.

14. Prior to April 1998, other researchers studied the effects of concentrations of ALA-esters that were about two orders of magnitude higher than the concentrations of the present invention. For example, Peng et al. "Build-up of esterified aminolevulinic-acid-derivative-induced porphyrin fluorescence in normal mouse skin" J. Photochem. Photobiol B: 34 (1996):95-96 (**Exhibit I**), administered methylester, ethylester and propylester of ALA in a concentration of 150 mg/kg, *i.e.*, about 20% (w/w). Our studies, based on a mean patient weight of 75 kg, administered a dose of approximately 1.3 mg/kg, which is about two orders of magnitude lower than that used in Peng et al.

15. Therefore, it was unexpected by me that the lower doses of ALA-esters would produce higher levels of PpIX than the lowest doses of ALA-esters studied at the time of the present invention.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the patent or any reexamination certificate issued therefor.

Dated: 26th of JANUARY 2004

G. Wagnières

GEORGES WAGNIÈRES, Ph. D.

October 2003

CURRICULUM VITAE: Georges Wagnières

PERSONAL INFORMATION

NAME:	Wagnières	FIRST NAME:	Georges
DATE OF BIRTH:	2nd May, 1962	NATIONALITY:	Swiss
MARITAL STATUS:	Married	CHILDREN	Three (8, 5 and 3 years)
PERMANENT HOME ADDRESS:	Chemin de Plantaz 56 CH-1095 Lutry Switzerland Tel.: + 41 21 791 13 43	CURRENT WORK ADDRESS:	LPAS Bâtiment de Chimie Swiss Federal Institute of Technology (EPFL) CH-1015 Lausanne Switzerland Tel.: + 41 21 693 31 20 Fax: + 41 21 693 36 26 e-mail: georges.wagnieres@epfl.ch

EDUCATION

Master in Management of Technology (January 2001 - December 2001)
Ecole des Hautes Etudes Commerciales (HEC) of the Lausanne's University / Swiss Federal Institute of Technology (EPFL): (Program comprising 550 hours of courses given in english in Lausanne, plus 60 hours given at the Business school of the University of Texas, Austin, USA. Moreover, this program involves an internship of 4 months).

Postdoctoral training (Research Fellow) (July 1993 - August 1994)
Harvard Medical School, Wellman Laboratories of Photomedicine, Boston, MA, USA.
Study: Characterization of photosensitizers and endogenous fluorophores in biological tissues by optical spectroscopy.

Doctorat ès Science (PhD) (May 1987 - April 1992)
Swiss Federal Institute of Technology (EPFL), Lausanne, Switzerland; Department of Physics;
Thesis topics: Photochemotherapy and photodetection of cancers using phototoxic and fluorescing agents.

Diplôme de physique (MS) (September 1981 - May 1986)
Lausanne University, Lausanne, Switzerland; Department of Physics.
Diploma topics: Radio Frequency Size Effect in Aluminium: Measurement of the Electrons Scattering Frequency.

Diplôme de culture générale scientifique (June 1979 - June 1981)
Gymnase Cantonal de la Cité, Lausanne, Switzerland.

PROFESSIONAL EXPERIENCE

From September 1994 to present
Appointed **'Adjoint scientifique' (Project Leader)** at the Swiss Federal Institute of Technology in Lausanne (EPFL), Switzerland. A position with research, teaching and management responsibilities.

- **Management activities:**
  - Responsible for the scientific and financial planning, as well as for the management of the "Cancer photodetection and photodynamic therapy (PDT)" group of the EPFL (14 coworkers).
  - Responsible for the obtaining of subsidies.
  - Creation and management of industrial and academic partnerships (about twenty institutions).
  - Responsible of the electronics and mechanics workshops of the laboratory (two technicians).
- **Teaching activities:**
  - Invited Professor at the University of Paris XIII, "Laboratoire de Physique des Lasers" (1998).
  - Supervision of 6 Ph.D. students.
  - Responsible of the diploma, basic and advanced physics laboratories performed in the PDT group (5 students/year).
- **Research Topics and technical skills.**
  - Preclinical and clinical evaluation of photosensitizers for photodynamic therapy.
  - Development of light distributors for endoscopic photodynamic therapy.
  - Study of the light dosimetry and of the tissue optical properties.
  - Clinical detection of early cancers by fluorescence imaging during endoscopy.
  - In vivo measurement of the vascular and tissular oxygen concentration using phosphorescing molecular probes.
  - Clinical tissue spectrofluorometry (steady-state and time-resolved) applied to: 1) the study of the pharmacokinetics of photosensitizers, 2) the spectral design of cancer photodetection apparatus.
  - Study of the histological and cellular localization of drugs by fluorescence microscopy.

From July 1993 to August 1994

Appointed Postdoctoral fellow at the **Harvard Medical School**, Wellman Laboratories of Photomedicine, Boston, MA, USA.

Research Topics and technical skills.

- Development of a microspectrofluorometer to measure quantitatively the fluorescence of endogenous and/or exogenous fluorochromes in histological sections.
- Study of a new approach to characterize biological tissues based on the use of environmentally sensitive fluorochromes.
- Preclinical study of the fluence rate effect and the photobleaching of photosensitizers.

Creation of collaborations.

- Prof. J. Fujimoto, Department of Electrical Engineering and Computer Sciences, Massachusetts Institute of Technology (MIT), Boston, MA, USA.
  Determination of the staging of superficial cancers by optical coherence tomography (OCT).
- Prof. B. Wilson and Prof. M. Patterson, Hamilton Regional Cancer Center, Ontario, Canada.
  Study of environmentally sensitive fluorochromes by frequency-domain time-resolved spectrofluorometry.

Teaching activity.

- Courses and seminars given in the tutorials program of the Wellman Laboratories. Subject: "Tissue characterization by fluorescence spectroscopy"

From May 1992 to June 1993

Same activity as between September 1994 and today (see above).

From May 1987 to April 1992

PhD student at the Swiss Federal Institute of Technology (EPFL), Lausanne, Switzerland.

Research: Photochemotherapy and photodetection of cancers using phototoxic and fluorescing agents.

ACHIEVEMENTS

ORIGINAL SCIENTIFIC REALIZATIONS:

- Development of instrumentations for the endoscopic detection of early superficial cancers and for their treatment by photodynamic therapy.
- Development of an original instrumentation to obtain fluorescence/phosphorescence lifetime images in real time and endoscopically in the frequency domain.
- Proposition of a novel method for the *in vivo* tissue characterization and cancer detection based on the use of fluorochromes sensitive to the physico-chemical properties of their environment. This principle is the basis technology of a start-up company (spin off of the Harvard Medical School).
- Development of a novel method to measure the tissue optical properties based on the use of the "CW spatial Fourier-transform reflectometry" approach.
- Application of optical coherence tomography (OCT) for the assessment of the staging of superficial carcinoma.
- Development of an experimental method to measure the fluorescence quantum yield of fluorophores in biological tissues.

CREATIONS:

- Co-founder and President of a spin-off biotech company (PhotoDerma SA; 1 coworker), founded in Mai 2003, which develops a technology for permanent hair removal.
- Co-founder and President of a spin-off biotech company (Medlight SA; 5 coworkers), founded in July 1997, which develops, produces and commercializes light distributors for photodynamic therapy.
- Significant contribution to the creation and financing of a multidisciplinary research group (Lausanne's PDT group) involving 15 coworkers active in the field of biomedical optics.

TRANSFER OF TECHNOLOGY:

- Most of our results and instrumental developments performed in the field of cancer detection by fluorescence imaging and photodynamic therapy have been transferred to medical instrumentation industries.
- The results of our studies regarding the optimization and assessment of new photosensitizers for photodynamic therapy have been transferred to several pharma companies.

INVENTIONS:

- Inventor of 10 patents (see the annex). Most of these patents are accepted at an international level. Three more patents are pending.

FINANCIAL SUPPORTS FOR ACADEMIC RESEARCH:

- More than 4'600'000 US$ obtained since 1993.
  - (60% from Swiss scientific research programs and Institutions; 15% from European Programs; 20% from Industries; 5% from Foundations).
  - (25% as unique applicant; 55% as co-applicant responsible for the scientific management; 20% as co-applicant).

LANGUAGES

FRENCH:	Fluently written and spoken (mother language)
ENGLISH:	Fluently written and spoken
GERMAN:	Working knowledge

COMPUTER SKILLS

Familiar with Apple and PC systems and office applications (Word, Endnote, Excel, Powerpoint, Acrobat, KaleidaGraph, Photoshop, NIH Image, Canvas, Eudora, Internet explorer, Netscape).

DISTINCTIONS

- Invited Professor at the University of Paris XIII (1998).
- Grant for advanced researcher provided by the Swiss national funds for scientific research; financial support for one year at the Harvard Medical School, Boston USA (1993 - 1994).
- Student award of the international photodynamic association, Buffalo, NY. (July 1990).

SOCIETIES

- The International Society for Optical Engineering
- Optical Society of America
- American Society for Photobiology
- European Optical Society
- Biomedical Optics Society
- American Society for Laser Medicine and Surgery
- The International Photodynamic Association
- Association Vaudoise des Chercheurs en Physique
- Société Suisse de Physique

BOARDS

- Topics Editor of "The Journal of Biomedical Optics".
- Chair of the Conference "Diagnostic Optical Spectroscopy", European Conference on Biomedical Optics, Munich, Germany; June 2003.
- President of the scientific committee and chairman of the "Troisième Colloque National OPT-DIAG '2000: "Optique pour le diagnostic Medical", Paris, France; May 2000.
- Chairman and member of the scientific committee of numerous international conferences.
- Evaluation of numerous grant requests addressed to financial sources (Pôles de Recherche Français, Fonds pour la recherche scientifique Belge, Netherlands Organisation for Scientific Research, Katholieke Universiteit Leuven).
- Referee for: Applied Physics B, Lasers in Medical Science; Optics Letters; Applied Optics; Photochemistry Photobiology; Gastroenterology; Progress in Biomedical Optics.
- Member of the scientific committee and chairman of numerous international conferences.
- Member of five foreign Ph.D. examining boards.

EXTRA-CURRICULAR ACTIVITIES

I enjoy outdoor activities namely skiing, jogging, volley-ball, sailing and mountain walking. Moreover, I appreciate family leisure. I have an interest for gastronomy and like to discover new cultures through traveling.

REFERENCES UPON REQUEST

ANNEX

PUBLICATIONS

Publications "Peer reviewed".

76) S. Andrejevic-Blant, A. Major, F. Lüdicke, J.-P. Ballini, G. Wagnières, H. van den Bergh and M.-F. Pelte, "Time-dependent hexyl-Aminolevulinic acid induced PPIX histopathologic distribution after topical application in patients with cervical intraepithelial neoplasia: A fluorescence microscopy study", submitted.

75) P. Wyss, R. Caduff, Y. Tadir, A. Degen, G. Wagnières, V. Schwarz, U. Haller, M. Fehr, "Photodynamic endometrial ablation: Morphological study", Lasers in Surgery and Medicine, 32(4), pp 305-309, 2003.

74) A. Degen, T. Gabrecht, L. Mosimann, M. Fehr, R. Hornung, V. Schwarz, Y. Tadir, R. Steiner, P. Wyss, "Photodynamic endometrial ablation for the treatment of dysfunctional uterine bleeding: A preliminary report", in press in: Am. J. Obstetrics & Gynecology.

73) A. Degen, T. Gabrecht, M. Fehr, G. Wagnières, R. Caduff, B. Imthurn, P. Wyss, "Influence of the menstrual cycle on the ALA induced protoporphyrin IX fluorescence in the endometrium: a clinical study", submitted.

72) Th. Stepinac, S. Chamot, E. Rungger, P. Ferrez, J.-L. Munoz, H. van den Bergh, C. Pournaraz, Ch. Riva, G. Wagnières, "Real-Time Monitoring of PDT-induced Retinal Vascular Damage by Phosphorescence Lifetime Imaging of a Pd-Porphyrin Oxygen Probe Used as Photosensitizer", submitted.

71) F. Lüdicke, T. Gabrecht, N. Lange, G. Wagnières, H. van den Bergh, L. Berclaz and A. Major, "Photodynamic diagnosis of ovarian cancer using aminolevulinic acid hexylester: A preclinical study", Brit. J. of Cancer, 88(11), pp 1780-1784, 2003.

70) F. Borle, A. Radu, G. Wagnières, H. van den Bergh, Ph. Monnier, "Evaluation of the photosensitizer Tookad for photodynamic therapy on the Syrian golden hamster cheek pouch model: Light dose, drug dose and drug-light interval effects", in press in: Photochem. Photobiol.

69) F. Borle, A. Radu, G. Wagnières, H. van den Bergh , Ph. Monnier "Evaluation of the photosensitizer Tookad on the Syrian golden hamster cheek pouch model: Response of photodynamic therapy on chemically induced squamous cell carcinoma versus healthy mucosae", in press in: Brit. J. Cancer.

68) S. Andrejevic Blant, Th. Glanzmann, J.-P. Ballini, G. Wagnières, H. van den Bergh, Ph. Monnier, "Uptake and localization of mTHPC (Foscan®) and its 14C-labeled form in normal and tumor tissues of hamster squamous cell carcinoma model: a comparative study", British Journal of Cancer, 87(12), pp 1470-1478, 2002.

67) A. Marti, P. Jichlinski, N. Lange, J.-P. Ballini, L. Guillou, F. Bosman, G. Wagnières, H. van den Bergh, P. Kucera, H.-J. Leisinger, "In vivo photosensitization of transitional cell carcinoma of the bladder with aminolevulinic acid or hexylester of aminolevulinic acid induced protoporphyrin IX", submitted.

66) A. Radu, R. Conde, G. Wagnières, Ch. Fontolliet, H. van den Bergh, Ph. Monnier, "Mucosal ablation with photodynamic therapy in the esophagus: Optimization of Light Dosimetry in the sheep model", in press in: Gastrointestinal Endoscopy.

65) R. Conde, Th. Glanzmann, A. Radu, J.-P. Ballini, H. van den Bergh, Ch. Fontolliet, Ph. Monnier and G. Wagnières, "Assessment of the sheep as a model to optimize photodynamic therapy with Foscan@ in the esophague", submitted.

64) M. Zellweger, D. Goujon, A. Radu, P. Grosjean, Th. Stepinac, H. van den Bergh, Ph. Monnier and G. Wagnières, "Detection of early bronchial cancer by autofluorescence: first clinical results with an improved system", submitted.

63) Th. Stepinac, Ch. Felley, P. Jornod, N. Lange, G. Wagnières, Ch. Fontolliet, H. van den Bergh, Ph. Monnier, P. Michetti, G. Dorta, "Endoscopic Fluorescence Detection of Dysplasia and Early Adenocarcinoma in Barrett's Esophagus after Administration of 5-Aminolevulinic Acid: a controlled Clinical Trial", Endoscopy, 35(8), pp 663-668, 2003.

62) D Goujon, M Zellweger, A Radu, P Grosjean, B-C Weber, H van den Bergh, Ph monnier, G. Wagnières. In vivo autofluorescence imaging of early cancers in the human tracheo-bronchial tree with a spectrally optimized system. J Biomed Opt, 8(1), pp 17-25, 2003.

61) Th. Stepinac, P. Grosjean, A. Woodtli, P. Monnier, H. van den Bergh, G. Wagnières, "Optimization of the Diameter of a Radial Irradiation Device for Photodynamic Therapy in the Esophagus", Endoscopy, 34(5), pp 411-415, 2002.

60) C. Wilder-Smith, P. Wilder-Smith, P. Grosjean, H. van den Bergh, A. Woodtli, Ph. Monnier, G. Dorta, F. Meister, G. Wagnières, "Photoeradication of Helicobacter pylori using 5-aminolevulinic acid: preliminary human studies", Laser in Surgery and Medicine, 31(1), pp 18-22, 2002.

59) G. Wagnières, A. Mcwilliams and S. Lam, "Lung cancer Imaging by autofluorescence bronchoscopy", in: Handbook of Biomedical Fluorescence, M.-A. Mycek and B. Pogue Editors, Marcel Dekker Inc., pp 361-396, 2002.

58) S. Andrejevic Blant, P. Grosjean, J.-P. Ballini, G. Wagnières, H. van den Bergh, Ch. Fontolliet and Ph. Monnier, "Localisation of tetra(m-hydroxyphenyl)chlorin (Foscan@) in human healthy tissues and squamous cell carcinomas of the upper aero-digestive tract, the esophagus and the bronchi: a fluorescence microscopy study", J. Photochem. Photobiol. B: biol., 61, pp 1-9, 2001.
57) N. Lange, J.-P. Ballini, G. Wagnières and H. van den Bergh, "A new Drug-Screening Procedure for Photosensitizing Agents used in Photodynamic Therapy of Choroidal Neovascularization", Investigative Ophthalmology & Visual Science, 42(1), pp 38-46, 2001.
56) M. Zellweger, P. Grosjean, D. Goujon, Ph. Monnier, H. van den Bergh and G. Wagnières, "Autofluorescence spectroscopy to characterize the histopathological status of bronchial tissue in vivo", J. of Biomedical Optics, 6(1), pp 41-52, 2001.
55) M. Zellweger, D, Goujon, M. Forrer, H. van den Bergh and G. Wagnières, "Absolute autofluorescence spectra of healthy bronchial tissue in vivo", Applied Optics, 40(22), pp 3784-3791, 2001.
54) Th. Glanzmann, M. Forrer, S. Andrejevic Blant, A. Woodtli, P. Grosjean, D. Braichotte, H. van den Bergh, Ph. Monnier and G. Wagnières, "Pharmacokinetics and Pharmacodynamics of tetra(m-hydroxyphenyl)chlorin in the Hamster Cheek Pouch Tumor Model: Comparison with Clinical Measurements", J. Photochem. Photobiol. B: Biol., 57, pp 22-32, 2000.
53) S. Andrejevic Blant, J.-P. Ballini, H. van den Bergh, Ch. Fontolliet, G. Wagnières and Ph. Monnier, "Time dependent biodistribution of tetra(m-hydroxyphenyl)chlorin and benzoporphyrin derivative monoacid ring A in the hamster model: comparative fluorescence microscopy study", Photochem. Photobiol., 71(3), pp 333-340, 2000.
52) M. Zellweger, A. Radu, Ph. Monnier, H. van den Bergh and G. Wagnières, "In vivo pharmacokinetics of Lutetium Texaphyrin in the healthy and tumoral cheek pouch mucosa. Retention and selectivity properties", J. Photochem. Photobiol. B: Biol., 55(1), pp 56-62, 2000.
51) A. Radu, G. Wagnières, H. van den Bergh, and Ph. Monnier, "Photodynamic therapy of early squamous cell cancers of the esophagus", Gastrointestinal endoscopy Clin North America, 10(3), pp 439-460, 2000.
50) R. Bays, A. Woodtli, L. Mosimann, P. Wyss, G. Wagnières, U. Haller, H. van den Bergh, "A light distributor for photodynamic endometrial ablation", Photomedicine in Gynecology and Reproduction; P. Wyss, Y. Tadir, B. Tromberg, U. Haller: Authors Editors, Karger, Basel, pp 227-233, 2000.
49) P. Uehlinger, M. Zellweger, G. Wagnières, L. Juillerat, H. van den Bergh and N. Lange, "5-Aminolevulinic acid and its derivatives: physical chemical properties and protoporphyrin IX formation in cultured cells", J. Photochem. Photobiol. B:Biol., 54, pp 72-80, 2000.
48) S. Iinuma, K. T. Schomacker, G. Wagnières, M. Rajadhyaksha, M. Bamberg, T. Momma and T. Hasan, "In vivo fluence rate and fractionation effects on tumor response and photobleaching: photodynamic therapy with two photosensitizers in an orthotopic rat tumor model", Cancer Res (United States), 59(24), pp 6164-6170, 1999.
47) J. Mizeret, Th. Stepinac, M. Hansroul, A. Studzinski, H. van den Bergh and G. Wagnières, "Instrument for real-time endoscopic fluorescence lifetime imaging", Rev. Sci. Instrum., 70(12), pp 4689-4701, 1999.
46) Th. Glanzmann, J.-P. Ballini, H. van den Bergh and G. Wagnières, "Time-resolved spectrofluorometer for clinical tissue characterization", Rev. Sci. Instrum., 70(10), pp 4067-4077, 1999.
45) M. Zellweger, P. Grosjean, Ph. Monnier, H. van den Bergh and G. Wagnières, "Stability of the fluorescence measurement of Foscan® in the normal human oral cavity as an indicator of its content in early cancers of the esophagus and the bronchi", Photochem. Photobiol, 69(5), pp 605-610, 1999.
44) N. Lange, P. Jichlinski, M. Zellweger, M. Forrer, A. Marti, L. Guillou, P. Kucera, G. Wagnières and H. van den Bergh, "Photodetection of early human bladder cancer based on the fluorescence of 5-aminolaevulinic acid hexylester-induced protoporphyrin IX: a pilot study", Brit. J. Cancer, 80(1/2), pp 185-193, 1999.
43) A. Radu, P. Grosjean, Ch. Fontolliet, G. Wagnières, A. Woodtli, H. van den Bergh and Ph. Monnier, "Photodynamic Therapy for 101 Early Cancers of the Upper Aerodigestive Tract, the Esophagus, and the Bronchi: A single-Institution Experience", Diagnostic and Therapeutic Endoscopy, 5, pp 145-154, 1999.
42) G. Wagnières, W. Star and B. Wilson, "In vivo fluorescence spectroscopy and imaging for oncological applications", Photochem. Photobiol., 68(5), pp 603-632, 1998.
41) G. Wagnières, Ch. Hadjur, P. Grosjean, D. Braichotte, J.-F. Savary, Ph. Monnier and H. van den Bergh, "Clinical evaluation of the cutaneous phototoxicity of 5, 10, 15, 20-tetra(m-hydroxyphenyl)chlorin", Photochem. Photobiol., 68(3), pp 382-387, 1998.
40) Ch. Hadjur, N. Lange, J. Rebstein, Ph. Monnier, H. van den Bergh and G. Wagnières "Spectroscopic studies of photobleaching and photoproduct formation of meta(tetrahydroxyphenyl)chlorin (mTHPC) used in photodynamic therapy. The production of singlet oxygen by mTHPC", J. Photochem. Photobiol. B: Biology, 45, pp 170-178, 1998.
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Oral presentations

Presentations given by G. Wagnières as invited speaker at international conferences (with financial support):

- "Instrumentation developped for the cancer photodynamic therapy in the upper aero-digestive tract", Meeting on PDT and Photodiagnosis: present state and future aspects, Meeting Hoffmann La Roche, Basel, July 1987.
- "Tissue characterization using environmentally sensitive fluorochromes", OE/LASE '94, Biomedical Optics, Los Angeles, USA, January 1994.

- "Measurement of the Photofrin II absolute fluorescence quantum yield in Tissue", 22nd Annual Meeting of the American Society for Photobiology, Scottsdale, Arizona, U.S.A., June 1994.
- "Les diffuseurs de lumière", Actualités en Thérapie Photodynamique, Paris, France, December 1996.
- "Clinical and Preclinical Measurements of fluorescence and photobleaching to optimize PDT and understand its kinetics", OE/LASE '97, International Biomedical Optics Symposium, San Jose, California, USA, February 1997.
- "Measurement of the benzoporphyrin derivative (BPD-MA) absolute fluorescence quantum yield in tissue", BiOS Europe '97, San Remo, Italy; September 1997.
- "Fluorescence lifetime imaging for endoscopic tissue characterization", 13th International Congress on Photobiology, Stresa, Italy, September 1997.
- "Clinical evaluation of the cutaneous phototoxicity of a second generation photosensitizer for PDT: mTHPC", PDT Physics Association Meeting, Scotia House, Stirling, Scotland, December 1997.
- "Laser tissue interaction: Photochemical effects", International Symposium on lasers in urology, Zürich, Switzerland; 4-6 March 1999.
- "Study of the tissue autofluorescence spectroscopy to optimize the detection of human bronchial precancerous and early cancerous lesions", Photodiagnosis and Photodynamic therapy in Clinical Practice, Innsbruck, Austria, 21-23 October 1999.
- "Tissue Fluorescence Spectroscopy", 7th United European Gastroenterology Week, Roma, Italy; 13-17 November 1999.
- "Basic principles of Photodynamic Therapy", 9th Congress of the European Academy of Dermatology & Venereology, Geneva, Switzerland; 11-15 October 2000.
- "Endoscopic tissue fluorescence spectroscopy to optimize the imaging photodetection of precancerous and early cancerous lesions", Photonics West, San Jose, USA; 19-25 January 2002.
- "Spectral Fluorescence Endoscopy", 12th World Congress for Bronchology, Boston, MA, USA; 16-19 June 2002.
- "La thérapie photodynamique: Principe, aspects instrumentaux et monitorage de la dose thérapeutique", XXIIIème Congrès de la SFLM, Arcs 1800, France; 26-29 January 2003.
- "First experience of hexyl-ester aminolevulinic acid induced fluorescence cystoscopy in patients with superficial bladder cancer", European Conference on Biomedical Optics, Munich, Germany; 22-25 June, 2003.
- "Detection of precancerous and early cancerous lesions in the bronchi by fluorescence/reflectance imaging with a spectrally optimized system", 10th Conference of the European Society for Photobiology, Vienna, Austria; 6-11 September, 2003.

Other oral presentations given by G. Wagnières at international conferences:

- "Instrumental aspects of light delivery for PDT in the upper aerodigestive tract", 2nd International Conference on Photodynamic Therapy and Medical Laser Applications, London, England, July 1988.
- "Detection of "Early" cancer in the upper aerodigestive tract and bronchi by fluorescence endoscopy: Apparatus and clinical results", 3rd International Conference on Photodynamic Therapy, Buffalo, USA, July 1990.
- "Measurement of the Photofrin II absolute fluorescence quantum yield in tissue", 4th International Conference on Photodynamic Therapy, Milan, Italy, June 1992.
- "Clinical evaluation of the photodamage to tumors, normal tissue and skin in PDT with mTHPC and Photofrin II", SPIE's international Symposium on Laser Engineering, Optoelectronic Packaging and Interconnects and Biomedical Optics, Los Angeles, U.S.A., January 1993.
- "Clinical measurements of tissue optical properties in the esophagus", BiOS Europe '94, International Symposium on Biomedical Optics, Lille, France, September 1994.
- "Clinical Measurement of Tissue Optical Properties in the Esophagus and in the Oral Cavity", 5th International Photodynamic Association Biennial Meeting, Amelia Island, Florida, USA, September 1994.
- "Caractérisation endoscopique de lésions cancéreuses précoces par spectrofluorométrie de fluorophores exogènes: applications cliniques", OPT-DIAG '95: Diagnostic et imagerie optiques en médecine, Paris, France, Mai 1995.
- "Measurement of the m-THPC absolute fluorescence quantum yield in tissue", European Biomedical Optics, BiOS Europe '95, Barcelona, Spain, September 1995.
- "Clinical Optimization in Photodynamic Therapy of early Squamous Cell Carcinoma located in the esophagus and the Tracheo-bronchial tree by endoscopic fluorescence spectroscopy", 6th Biennial Meeting of the International Photodynamic Association, Melbourne, Australia, March 1996.
- "Design and characterization of a phantom simulating the tissue optical properties over the visible part of the spectrum", BiOS Europe '96, Vienna, Austria, September 1996.
- "Clinical evaluation of the cutaneous phototoxicity of mTHPC", Seventh Biennial Congress of the International Photodynamic Association, Nantes, France; 7-9 July, 1998.
- "Clinical study of the mTHPC-induced tissue photosensitization under various conditions", BiOS Europe '98: Norra Latin Conference Center Stockholm, Sweden; 8-12 September 1998.
- "Frequency-domain fluorescence lifetime imaging (FLIM) for real-time endoscopy tissue characterization" CLEO/Europe, Münich, Germany; 13-16 June 1999.
- "Study of the tissue autofluorescence spectroscopy to optimize the detection of human bronchial precancerous and early cancerous lesions", EBiOS 2000: The free University of Amsterdam, The Netherlands, 4-8 July 2000.
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   U.S. Patent: 4,998,797
   European Patent: 0413660
   Inventors: H. van den Bergh, G. Wagnières
2) Apparatus for irradiating the bronchi of a patient for the purpose of photodynamic therapy.
   U.S. Patent: 5,054,867
   European Patent: 0437181
   Inventors: G. Wagnières, H. van den Bergh, Ph. Monnier
3) Apparatus for homogenising the non-homogeneous light distribution of a laser beam.
   U.S. Patent: 5,068,515
   European Patent: 0435825
   Inventors: H. van den Bergh, P. Cornaz, G. Wagnières
4) Device for injecting the light energy of a laser beam into a fibre-optical waveguide and a method for adjusting and monitoring the position of the end of the fibre-optic optical waveguide.
   U.S. Patent: 5,117,474
   European Patent: 0421929
   Inventors: H. van den Bergh, P. Cornaz, G. Wagnières
5) Fibre-optic apparatus for the photodynamic treatment of tumours.
   U.S. Patent : 5,146,917
   European Patent: 0437182
   Inventors: G. Wagnières, H. van den Bergh, Ph. Monnier
6) Light diffuser for the photodynamic therapy of tumours in the oesophagus of a patient.
   U.S. Patent: 5,219,346
   European Patent: 0437183
   Inventors: G. Wagnières, H. van den Bergh, Ph. Monnier
7) Device for irradiating internal cavities of the body

WO9911322
Inventors: R. Bays, A. Woodtli, G. Wagnières, H. van den Bergh

8) Diagnosis apparatus for the picture providing recording of fluorescing biological tissue regions.
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Inventors: G. Wagnières, M. Zellweger, N. Chauvin, N. Lange, U. Zanger, A. Studzinski, H. van den Bergh

9) Pharmaceutical use of 5-aminolevulinic acid ester solution
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Inventors: P. Eidner, T. Goll, S. Müller, N. D. Pereira, O. Schmidt, B. C. Weber, H. van den Bergh, D. Goujon, G. Wagnières

11) Hair removal by PDT with derivatives of ALA
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Inventors: G. Wagnières, N. Lange, N. Doegnitz, D. Salomon, H. van den Bergh

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J. Photochem. Photobiol. B: Biol. 54 (2000) 72–80

Journal of Photochemistry and Photobiology B:Biology

5-Aminolevulinic acid and its derivatives: physical chemical properties and protoporphyrin IX formation in cultured cells

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Received 29 March 1999; accepted 8 December 1999

Abstract

Protoporphyrin IX (PpIX) is used as a fluorescence marker and photosensitizing agent in photodynamic therapy (PDT). A temporary increase of PpIX in tissues can be obtained by administration of 5-aminolevulinic acid (ALA). Lipophilicity is one of the key parameters defining the bioavailability of a topically applied drug. In the present work, octanol–water partition coefficients of ALA and several of its esters have been determined to obtain a parameter related to their lipophilicity. The influence of parameters such as lipophilicity, concentration, time, and pH value on PpIX formation induced by ALA and its esters is then investigated in human cell lines originating from the lung and bladder. ALA esters are found to be more lipophilic than the free acid. The optimal concentration (c_{opt}, precursor concentration at which maximal PpIX accumulation is observed) is then measured for each precursor. Long-chained ALA esters are found to decrease the c_{opt} value by up to two orders of magnitude as compared to ALA. The reduction of PpIX formation observed at higher concentrations than c_{opt} is correlated to reduced cell viability as determined by measuring the mitochondrial activity. Under optimal conditions, the PpIX formation rate induced by the longer-chained esters is higher than that of ALA or the shorter-chained esters. A biphasic pH dependence on PpIX generation is observed for ALA and its derivatives. Maximal PpIX formation is measured under physiological conditions (pH 7.0–7.6), indicating that further enhancement of intracellular PpIX content may be achieved by adjusting the pharmaceutical formulation of ALA or its derivatives to these pH levels.

Keywords: 5-Aminolevulinic acid; 5-Aminolevulinic acid esters; Photodynamic therapy (PDT); Fluorescence; Protoporphyrin IX; ALA esters; Lipophilicity

1. Introduction

The exogenously stimulated formation of intracellularly generated protoporphyrin IX (PpIX), a precursor of heme, is becoming one of the fastest developing areas in the field of photodynamic therapy (PDT) and fluorescence photodetection (PD) of malignant and non-malignant diseases (see Ref. [1] and references therein). In most clinical and preclinical studies, systemic or topical application of 5-aminolevulinic acid (ALA) is used to temporarily increase the concentration of PpIX in the target tissues. Administration of ALA, a metabolic precursor in the biosynthetic pathway of heme, bypasses the negative feedback control exerted by heme on the enzymatic step in ALA synthesis. Although PpIX formation is present in nearly every nucleated cell, preferential formation and accumulation of this photosensitizer have been demonstrated in tissues known to have a high cellular turnover. The main reason for a somewhat selective PpIX accumulation in the latter cell types is still not completely understood. Experimental evidence has been found that, in some tumors, the ferrochelatase activity is reduced, while the activity of the porphobilinogen deaminase is enhanced [2,3]. For historical reasons [4] and due to the ease of administration to the skin of both drug and light, the main applications of ALA-mediated PpIX therapy are in dermatology. This modality is now in Phase III trials for the treatment of actinic keratosis and has also been employed clinically for the treatment of basal cell carcinoma. Recently, other medical fields, namely pulmonology [5], urology [6–9], gastroenterology [10], ENT [11], gynecology [12], and neurosurgery [13], have implemented this technique for the improved management of cancer. In addition to its tumor selectivity, the administration of ALA prevents prolonged cutaneous photosensitivity, one of the major drawbacks of some of the earlier photosensitizers [14].

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1011-1344/00/$ - see front matter
PII S1011-1344(99)00159-1

Despite promising results, it appears that this methodology is open to quite significant improvement, in particular in the case of topically applied ALA. Since ALA is a hydrophilic molecule, its penetration through cellular membranes and into the interstitial space of tissues is low. Hence, ALA-induced PpIX formation is often limited to superficial tissue layers. Furthermore, PpIX formation shows considerable heterogeneity when ALA is applied topically. Both inhomogeneous and limited tissue distribution result in nonefficient treatment of deeper-lying or nodular lesions, even if light in the red region of the PpIX absorption spectrum is used [15,16]. Since deeper-lying lesions are often not accessible by PDT, they are missed after topical application of ALA. Consequently, relatively high doses of ALA have to be applied over long periods of time, increasing the risk of complications [17,18].

Due to these drawbacks, PpIX-mediated PDT and diagnosis have recently been started with more lipophilic derivatives of ALA in order to enhance the poor bioavailability of ALA. Several groups have shown that using such ALA prodrugs may enhance the PpIX concentration by up to two orders of magnitude as compared with the parent molecule [19–23].

Since lipophilicity is one of the key parameters, in the present study the octanol–water partition coefficient P of some alkyl esters has been determined as a measure related to this property. With the final goal of defining clinical protocols with improved bioavailability of ALA, we investigated the impact of lipophilicity, pH value, concentration and duration of exposure of ALA and its derivatives on PpIX formation and cell viability. This was performed by means of fluorescence spectroscopy of PpIX using four different human cell lines. It was demonstrated that long-chained ALA derivatives and physiological pH values resulted in the highest relative fluorescence values. Using ALA derivatives, the choice of the optimal concentration of the PpIX precursor was shown to be of major importance for cell viability and maximal PpIX formation.

2. Materials and methods

2.1. Chemicals

ALA hydrochloride, ALA-methylester hydrochloride (m-ALA), (3-4,5-dimethylthiazol-yl)-2,5-diphenyl tetrazolium bromide (MTT), and *n*-octanol were purchased from Sigma (Fluka, Buchs, Switzerland). 0.1 N NaOH was obtained from Merck (Darmstadt, Germany). Other ALA esters (Table 1) were synthesized in our laboratories following the procedure described recently by Kloek et al. [20].

2.2. Determination of physicochemical properties

The apparent partition coefficients (P) of ALA and its esters were determined in an octanol–buffer system at 21°C.

Table 1
List of hydrochlorides of ALA and esters used for in vitro experiments ($HCl \cdot R^2{}_2N–CH_2–CO–CH_2–CH_2CO–OR^1$ = general structure)

Compound	R^1	R^2	Mol. mass [g/mol]	Abbreviation
ALA	H	H	167.6	ALA
ALA-methylester	CH_3	H	181.6	m-ALA
ALA-ethylester	CH_2CH_3	H	195.6	e-ALA
ALA-butylester	$(CH_2)_3CH_3$	H	223.8	b-ALA
ALA-hexylester	$(CH_2)_5CH_3$	H	251.8	h-ALA
ALA-octylester	$(CH_2)_7CH_3$	H	279.6	o-ALA
ALA-cyclohexylester	C_6H_{11}	H	249.8	ch-ALA

The aqueous phase was a 0.1 M phosphate buffer (PBS) solution of pH 7.4. The PBS solution and the octanol were mutually saturated before use by shaking 300 ml of PBS with an equal quantity of octanol for 30 min. Twenty milligrams of the compound to be investigated were dissolved in 10 ml of the aqueous phase and an equal quantity of octanol was added. The mixtures were shaken for about 30 min and left for phase separation overnight at 4°C. Absorption of both phases was measured with a UV–Vis absorption spectrometer (Cary 5, Varian, Australia) at 269 nm (see Fig. 1(a)). The partition coefficients P were calculated according to:

$$P = c_{oct}/c_{PBS} = abs_{oct}/abs_{PBS}$$

where c_{oct} and c_{PBS} represent the solute concentrations in the organic and the aqueous phase, respectively, abs_{oct} the absorption of the compound measured in the octanol and abs_{PBS} the absorption in the PBS solution (see Fig. 1(b)). The use of low concentrations and storage at low temperatures impaired the formation of dimerization products. The absence of these products was confirmed by the absence of characteristic absorption bands in the absorption spectrum of the measured solutions.

Values of acidity constants of ALA and its esters were measured by means of potentiometric titration with a standard

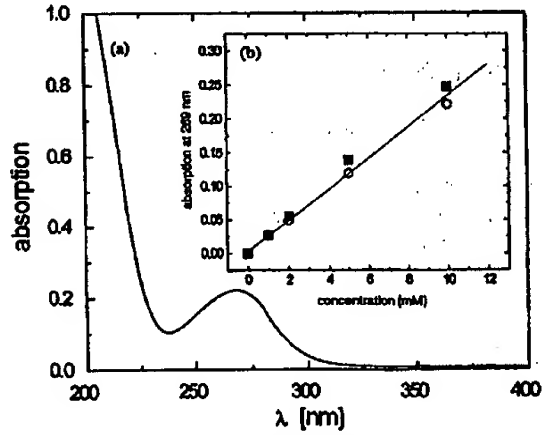

Fig. 1. (a) Absorption spectrum of b-ALA (10 mM) in PBS. (b) Absorption at 269 nm as a function of b-ALA concentration, (■) in PBS, (○) in octanol.

pH electrode (Bioblock, Prenkendorf, Switzerland). In brief, 20 mg of the corresponding drug were dissolved in 10 ml of demineralized water and titrated with 0.1 N NaOH solution. The pH of the solution was plotted against the total volume added.

2.3. PpIX fluorescence measurements

For fluorescence measurements, cells, subcultured in 48-well dishes, were exposed to various concentrations of the corresponding PpIX precursor and transferred into a thermostated fluorescence multiwell plate reader (CytoFluor Series 4000, PerSeptive Biosystems, Framingham, MA, USA, excitation wavelength $\lambda = 409 \pm 10$ nm, detection wavelength $\lambda = 640 \pm 20$ nm). Correction for cell autofluorescence and other offset parameters was provided by five wells not exposed to the PpIX precursors. Reference was provided by 200 μl of a Rhodamine 6G (0.1 g/l) (Lambda Physik, Göttingen, Germany) solution always present in one of the dishes.

2.4. Cell cultivation

All cell lines were from ATCC (Rockville, MD, USA) and grown as described. J82 and T24 cells were derived from human transitional cell carcinoma of the bladder, A549 cells from human lung carcinoma, and BEAS-2B cells were immortalized from normal human bronchial epithelium. Culture was performed in the presence in 10% fetal calf serum (FCS) and penicillin–streptomycin at 37°C and 6% CO_2 in a humid environment. For measurement purposes, the cells were subcultured in 48-well dishes (Costar 3548, Integra Biosiences, Cambridge, MA, USA) to give 10^5 cells/well 72 h prior to incubation with the ALA or one of its derivatives.

2.5. Determination of cell viability

The cell viability was tested by means of an MTT assay. This technique allows quantification of cell survival after cytotoxic insult by testing the enzymatic acitivity of the mitochondria. It is based on the reduction of the water-soluble tetrazonium salt to a purple, insoluble formazan derivative by mitochondrial enzyme dehyrogenases. This enzymatic function is only present in living, metabolically active cells. The optical density of the product was quantified by its absorption at 540 nm using a 96-well ELISA plate reader (iEMS Reader MF, Labsystems, USA). MTT, 0.5 mg/ml, was added to each well and incubated for 2 h at 37°C. The medium was then removed and the cells were washed with PBS solution. For cell lysis and dissolution of the formazan crystals formed, 250 μl of isopropanol containing 1% 4 N HCl were added, and the absorption of each residue was determined by using the plate reader at 540 nm. Absorbance of the solution from cells incubated with ALA or its derivatives was divided by the absorption of the solution from the control cell plates to calculate the fraction of surviving cells.

2.6. Concentration and time dependence of PpIX formation

The influence of precursor concentration on the total amount of PpIX formed was measured by permanent incubation of the different cell lines with a given ALA derivative dissolved in PBS at pH 7.4. PpIX was measured 3 and 6 h after drug exposure. Concentration-dependent saturation of PpIX biosynthesis in A549 cell cultures was determined by using different concentrations (0.1–2 mM) of h-ALA. Cells were incubated with a medium containing 5% FCS and fluorescence measurements were carried out every 30 min during 24 h. The influence of the presence of FCS on the PpIX formation was examined by incubation of A549 cells with a 0.8 mM solution of h-ALA containing no, 1%, and 5% FCS, respectively. In order to correct all data for background autofluorescence, in each experiment six wells were incubated without any PpIX precursor.

2.7. Pharmacokinetic studies

PpIX formation in cells incubated with different derivatives of ALA was followed over a period of 5 h. For this purpose, the cells were incubated with the corresponding PpIX precursor at its optimal concentration (as determined according to the above-mentioned procedure). Measurements of fluorescence intensity were taken every 15 min.

2.8. pH Dependence of PpIX formation

The impact of initial extracellular pH was determined using solutions of ALA, h-ALA, and ch-ALA in sterile, isotonic NaCl (aq., 0.9%). The initial pH values, ranging between 5.5 and 8.5, were adjusted with 1 N NaOH for ALA and 0.1 N HCl for ALA derivatives. ALA and its derivatives were applied using concentrations lower than the optimal concentration, typically $c_{opt}/2$. Fluorescence intensity was measured immediately after incubation and again after 3 h. Cell viability was tested as described in Section 2.5.

3. Results and discussion

3.1. Physicochemical properties

The lipophilicity of ALA and its derivatives was assessed by measuring the apparent partition coefficient (P) of the compounds between octanol and a PBS solution of pH 7.4. Table 2 summarizes the obtained log P values. The results plotted in Fig. 2 show that it is possible to vary the lipophilicity of ALA by more than three orders of magnitude when using ALA esters. The log P values of ALA and m-ALA are negative, representing the hydrophilic feature of these substances. Relative to ALA and m-ALA, all other esters are more lipophilic with positive log P values. Both ALA and its esters are highly protonated at pH 7.4 due to the 5-amino group. Therefore the apparent partition coefficient may be

Table 2
Log P and pK_a values for ALA and its derivatives. P is the partition coefficient between octanol and aqueous buffer solution (pH 7.4, 21°C)

Compound	log P	pK_{a1}	pK_{a2}
ALA	−1.51692	4.1 ± 0.1	8.7 ± 0.2
m-ALA	−0.94233		8.4 ± 0.3
e-ALA	0.84113		8.4 ± 0.2
b-ALA	1.42315		8.3 ± 0.1
h-ALA	1.83883		8.3 ± 0.3
o-ALA [a]	2.6199		
ch-ALA	1.49392		8.3 ± 0.2

[a] pK_{a2} not measurable because of precipitation.

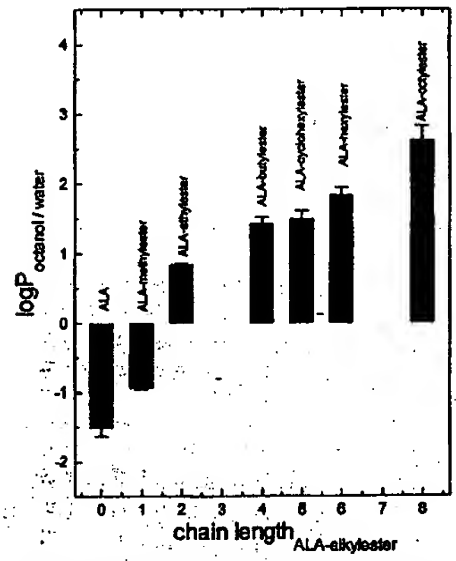

Fig. 2. Derived log P values for ALA and its derivatives.

dependent on the relative amount of uncharged molecules in the aqueous phase. The percentage of molecules with an unprotonated amino group can be calculated using the measured pK_a values (see Table 2). For the different derivatives, this percentage varies between 6 and 12%. The pK_{a1} and pK_{a2} values for ALA given in Table 2 are in good agreement with the results of Novo et al. [24]. The deprotonation of the amino group in o-ALA results in a reversible precipitation of the product in aqueous solution at pH ~ 8 and at a concentration around 20 mM. Furthermore, cleavage of some esters occurs in basic (pH > 9) solutions (data not shown) [25].

Systematic studies of Bridges et al. [26] with a series of homologue carbamates have shown a relatively constant absorption rate for compounds with log P values ranging between 0.8 and 2.8. However, carbamates with log P values less than 0.8 have shown reduced bladder-wall absorption. This suggests a higher tissue uptake for ALA esters containing two or more carbon atoms in their ester function.

Besides higher solubility of compounds with higher lipophilicity in creams and ointments, the data presented in Table 2 have additional impact for the use of ALA esters in dermatology. One of the principal functions of the skin, in particular the stratum corneum (SC), is to avoid the absorption of compounds that come in contact with the skin's surface. Using an approximation based on the analysis of 90 compounds [18], one can estimate the steady-state permeability coefficient K_p of ALA derivatives. It can be calculated that b-ALA will be transported about 50 times more efficiently into the skin than ALA while, using m-ALA, this uptake rate will only be doubled. However, the magnitude of P is important in terms of drug bioavailability. Substances that are too lipophilic may be accumulated in the SC, which consists primarily of free fatty acids, cholesterol, and ceramides. In order to obtain maximal flux across the entire skin, a balanced partition coefficient and good water and lipid solubility are required. Furthermore, the intrinsic solubility may be modified by co-diffusing formulation components.

Additionally, one should bear in mind that facilitated drug uptake does not automatically mean higher PpIX formation. Esters of ALA must be cleaved by esterases before entering the ordinary biosynthetic pathway of heme. These enzymes may have a more or less marked affinity to certain ester functions [21].

3.2. Influence of concentration on PpIX accumulation

The amount of porphyrin biosynthesis resulting from incubation of cells with ALA or its derivatives was determined by measuring the intensity of PpIX fluorescence.

All cell lines displayed the capability to produce PpIX when exposed to ALA or a prodrug given in Table 1. Since FCS has been shown to provoke efflux of PpIX in several cell lines [27], we incubated A549 cells with h-ALA for 5 h with and without FCS. Under our experimental conditions no influence of FCS on the total amount of PpIX generated has been found (see Table 3). The effect of concentration was assessed using ALA or ALA prodrug concentrations varying over two orders of magnitude. As shown in Fig. 3, there was a dose-dependent PpIX accumulation for each cell line and for each PpIX precursor used. The shape of the dose–response curves was similar in each case. While PpIX generation is positively correlated up to an optimal prodrug concentration (c_{opt}) where the highest PpIX fluorescence levels occurred, PpIX generation decreases when this threshold concentration is exceeded. The absolute value of the optimal concentration varies with the type of prodrug and cell line. Except for the

Table 3
Influence of FCS on the PpIX formation in A549 cells after 5 h of incubation with 0.8 mM of h-ALA

FCS (%)	PpIX fluorescence [a.u.]
0	680 ± 150
1	670 ± 170
5	680 ± 140

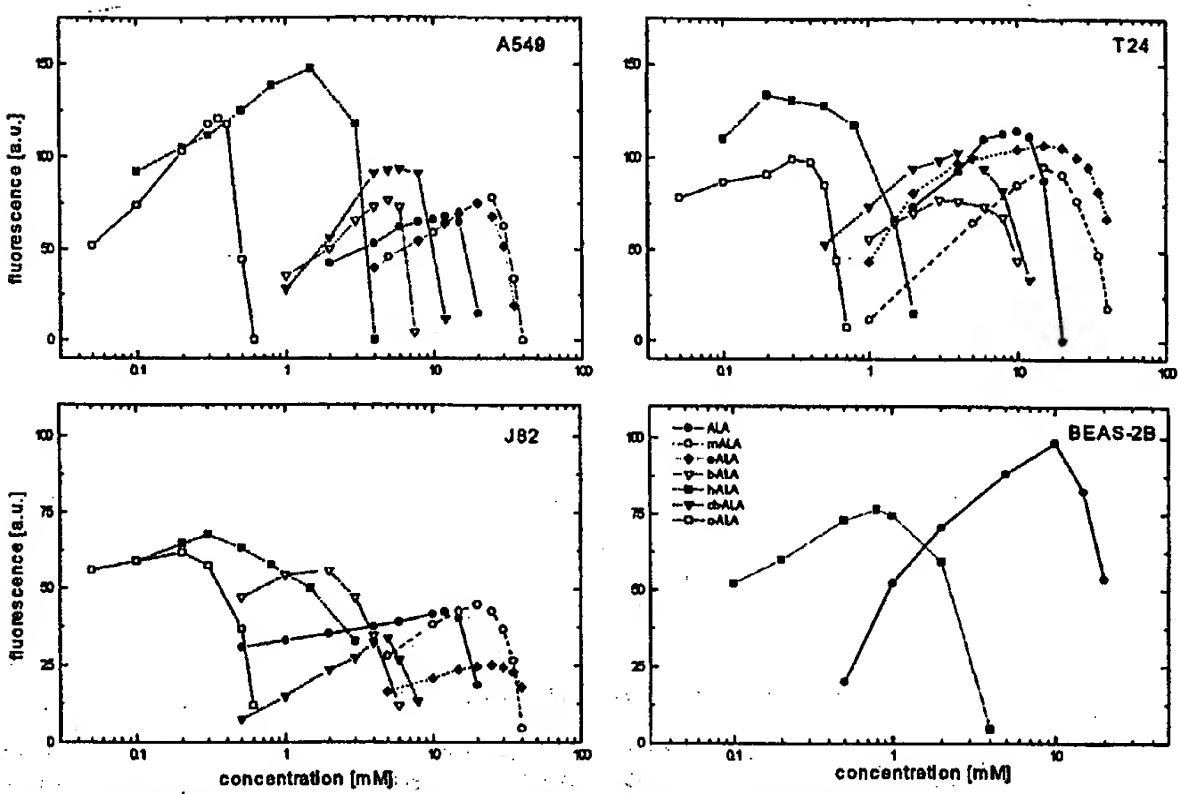

Fig. 3. Concentration dependence of PpIX accumulation for four different human cell lines after 3 h of incubation with ALA (●), m-ALA (○), e-ALA (♦), b-ALA (△), h-ALA (■), ch-ALA (▼), and o-ALA (□) (standard deviations (SD) have been omitted for the sake of clarity, see Fig. 5 for exemplary SD).

BEAS-2B cell line, incubation with h-ALA resulted in the highest fluorescence levels.

In general, the c_{opt} values for m-ALA and e-ALA were higher than for ALA. ALA esters with alkyl groups consisting of four carbon atoms or more (b-ALA, h-ALA, and o-ALA) showed their optimal PpIX formation at significantly lower c_{opt} values (Fig. 4). Furthermore, the bandwidth of the dose–response curves for these esters was always smaller than for ALA, m-ALA, or e-ALA (Fig. 3), indicating that the choice of the optimal concentration is crucial in order to guarantee an optimal PpIX generation.

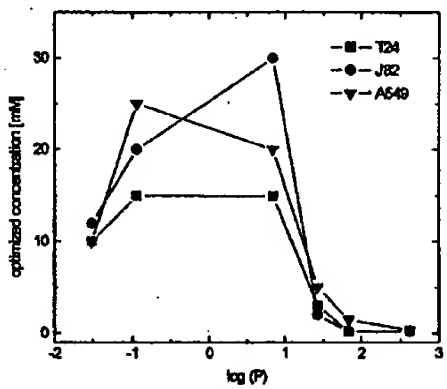

Fig. 4. Concentration of ALA and its derivatives needed to induce maximal PpIX accumulation after 3 h of incubation on A549 (▼), T24 (■), and J82 ( ) cell lines. See also Table 3.

Similar fluorescence intensity–concentration profiles were measured after 300 min of incubation (data not shown). Both the value of c_{opt} and the bandwidth of the dose–response curves remained unchanged. The long-term influence of permanent drug exposure on PpIX biosynthesis was tested by incubation of A549 cells with h-ALA, which has shown the most promising results under our conditions with respect to its dose–response behavior (Fig. 5). For concentrations smaller than c_{opt}, PpIX formation increases in a moderate sigmoidal way with incubation time (Fig. 5(a)). Under optimal conditions, continuously increasing PpIX accumulation can be observed for 24 h. Depending on the concentration, the linear part of these curves ends between 4 and 15 h and proceeds into a moderate plateau. For small concentrations the height of this plateau depends linearly on the concentration of h-ALA, whereas higher concentrations show a saturation of the PpIX biosynthesis (Fig. 5(b)). This might indicate a saturation of the enzymatic functions. However, higher concentrations than c_{opt} end with less PpIX formed, although no reduced cell viability has been determined under these conditions (see below).

No direct correlation between log P and c_{opt} or the amount of PpIX produced can be noted from the data in the present work (Fig. 4). Excluding ch-ALA, however, a decrease of

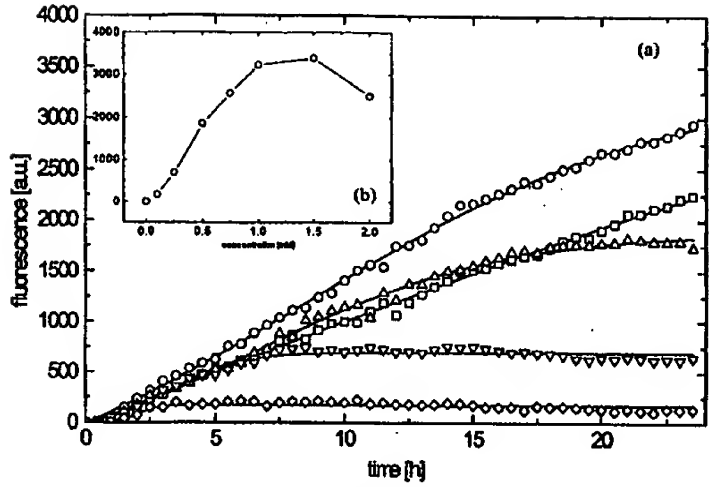

Fig. 5. (a) Pharmacokinetics of PpIX synthesis in A549 cells treated with different concentrations of h-ALA during 24 h of permanent drug exposure. Concentrations: 2 mM (□), 1.5 mM (○), 0.5 mM (△), 0.25 mM (▽), and 0.1 mM (◇). (b) PpIX formation after 24 h as a function of concentration of h-ALA solutions (for description see Section 2).

c_{opt} with increasing chain length for lipophilic ALA derivatives (starting from e-ALA) can be established.

While long-chained derivatives ($C \geq 4$) showed lower c_{opt} values than ALA, m-ALA as well as e-ALA seemed to be less efficient, although more lipophilic than ALA. The combination of two distinct processes may explain this behavior. While lipophilicity defines the transport of a drug across cell membranes, ALA esters must be cleaved by nonspecific esterases prior to entering the biosynthetic pathway of heme. Kloek et al. [21] have shown with cell lysates that enzymatic hydrolysis is faster for long-chained esters than for short-chained esters. This information might have an impact for further synthesis of derivatives of ALA. Such new prodrugs should have a similar lipophilicity to h-ALA or o-ALA and the enzymatic cleavage of the ester function should also be optimized. Derivatives of ALA can be adapted to specific esterases of tumor cells for further improvement of the selectivity of ALA-induced PpIX.

Similar dose-dependence characteristics have also been observed by other groups with ALA [28–32]. These groups found either a saturation of PpIX or a slight decrease of the resulting PpIX fluorescence with increasing ALA concentration. Gaullier et al. [22] observed an optimal concentration on different human and animal cell lines for long-chained ALA esters in the same order of magnitude as presented in this work. The more than twofold increase of the PpIX formation rate with ALA esters as compared with ALA is in good agreement with the results we recently obtained from measurements on an organ culture model [19]. Recently, Kloek et al. [21] compared the performance of different ALA derivatives on human lymphoma cell lines. They found that ALA pentyl ester induced the highest PpIX levels in intact cells, while h-ALA and b-ALA have shown similar fluorescence intensities after 6 h of incubation. However, in the course of their experiments, incubation was performed using equimolar concentrations for all derivatives. Hence, it might be possible that for long-chained alkyl esters the concentration was too high to produce large amounts of PpIX.

The relative rate of PpIX generation increases with increasing lipophilicity of the corresponding ALA ester from m-ALA to h-ALA, whereas comparable rates of h-ALA and o-ALA suggest a saturation of some enzymatic functions in the biosynthetic pathway within these time ranges. Taking into account c_{opt}, which was 10–100 times lower for long-chained ALA esters than for ALA, it can be concluded that, using such compounds, the PpIX formation efficiency was enhanced by almost two orders of magnitude by simple chemical derivatization.

Since ALA is known to induce cytotoxic effects in cell culture [33–35], the PpIX accumulation observed was evaluated with respect to the cell viability after incubation with different concentrations of each PpIX precursor. It can be seen from Fig. 6 that the reduction of PpIX formation after incubation with drug doses higher than c_{opt} coincides with a reduced cell survival. This correlation was observed for all cell lines and prodrugs. Incubation with lower doses of ALA or its derivatives did not affect cell viability, as confirmed by the MTT test. From these experiments, it is obvious that only well-defined drug doses will improve the PpIX formation in clinical applications when using ALA esters instead of ALA.

3.3. pH Dependence of PpIX formation

It has been found that tumor tissues are generally more acidic than surrounding normal tissues. This is probably due to an overproduction of lactic acid and hydrolysis of adenosine triphosphate (ATP). Since this microenvironmental factor may influence PpIX generation, we incubated three cell lines with ALA, ch-ALA, and h-ALA solutions adjusted to pH values in the range between 5.5 and 8.5. In order to prevent

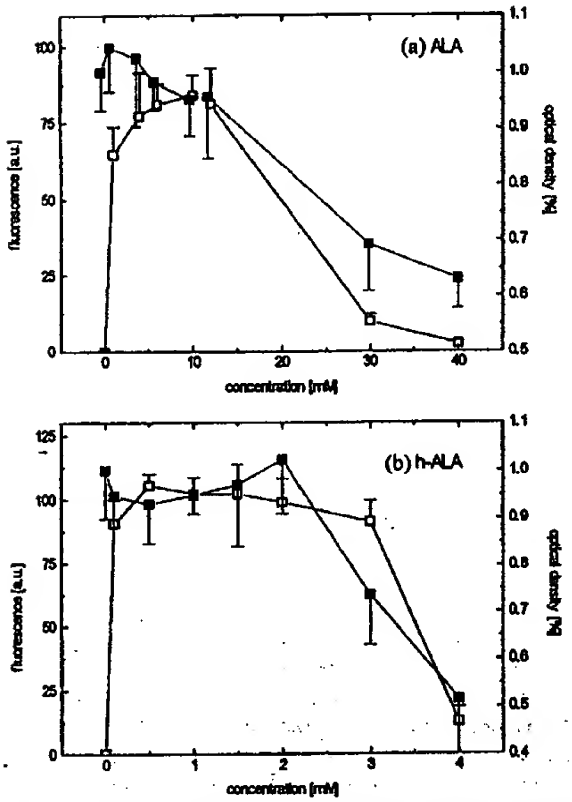

Fig. 6. Correlation of PpIX fluorescence intensity (□) and mitochondrial activity test by means of an MTT assay (■) in A549 cell for (a) ALA and (b) h-ALA (for description see Section 2).

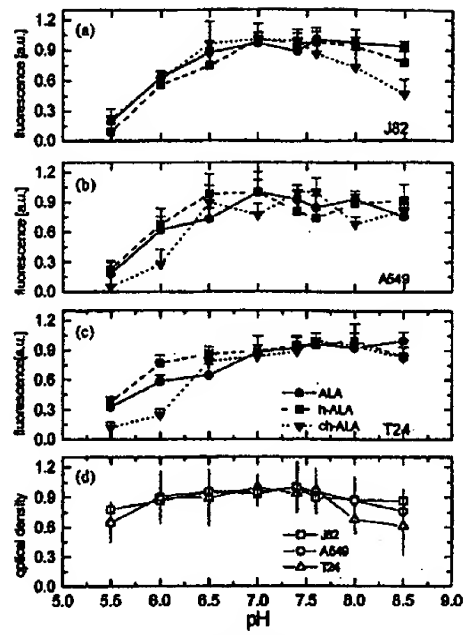

Fig. 7. PpIX formation as a function of initial pH values in (a) J82, (b) A549 and (c) T24 cell lines after 120 min of incubation with 0.1 mM h-ALA (■), 3.2 mM ch-ALA (▼), and 5 mM ALA (●) solutions (fluorescence values are normalized to the maximum of fluorescence). (d) Mitochondrial activity test as a function of initial pH values.

saturation or cytotoxic effects provoked by the drug itself, concentrations lower than c_{opt} ($c_{opt}/2$) were chosen. The values plotted in Fig. 7 indicate that optimal PpIX formation occurs at physiological pH values of around 7.5 ± 0.5. The total PpIX production approximately tripled at pH 7.4 compared with the production induced at pH 5.5. Due to proton release to the nonbuffered medium, the initial pH values decreased during incubation. While under alkaline conditions this effect was more marked ($\Delta pH \sim 0.3/h$ at 8.5), the pH values under acidic conditions remained nearly unchanged ($(\Delta pH \sim 0.01/h$ at pH 6.5)). Generally, PpIX production was more drastically reduced under acidic than under alkaline conditions, extending previously published results using ALA as a PpIX precursor [28,36,37]. While the decrease of PpIX formation at higher pH values can be attributed to a reduction of cell viability, the decrease under acidic conditions can be attributed to either a pH-dependent drug uptake or a reduced enzymatic activity in the biosynthesis of heme. As has been shown, ALA uptake is regulated by a pH-dependent ion pump that is more active at pH 5.0 [38]. Hence, PpIX production would be expected to increase under acidic conditions. However, it is known that intracellular pH is also downregulated when extracellular pH falls below 6.5 [39]. This might inhibit the activity of enzymes involved in the biosynthetic pathway of heme, which have optimal activity between pH 7 and 7.5 [37]. A further indication for this pH-dependent intracellular process is given by the use of ALA esters, since a major part of these amphiphilic compounds will be taken up actively, as demonstrated by inhibitory tests [40].

From these experiments, it can be concluded that, for diagnostic as well as for therapeutic reasons, ALA formulations adjusted to physiological pH values should be applied. However, the instability of ALA implies the administration of ALA solution adjusted to lower pH values for the photodetection of early human bladder cancer in urology [6–9,24]. Since these are physiological pH values for urothelial cells, the uptake of ALA may not be affected by solutions buffered to a pH of 5. In contrast, the production of PpIX under these conditions may be strongly dependent on this parameter. Novo and colleagues [24] have attributed the chemical instability of ALA to an irreversible dimerization of two parent molecules followed by an oxidation of the resulting dihydropyrazine derivative. Generally, the velocity of such bimolecular reactions is proportional to the product of the concentrations of the two involved reactants. Hence, under this assumption the drastic reduction of the concentration by a factor of about 20 [22] that is used with ALA esters enhances the stability of the corresponding solution by a factor of 400. This increase of stability opens the possibility for a further increase of PpIX formation after topical application of ALA derivatives by a simple adjustment of the pharmaceutical formulation to physiological pH values.

4. Conclusions

In summary, this study shows that using esters of ALA instead of ALA indicates a promising route to improve many clinical applications of PpIX-mediated PDT and fluorescence photodetection. The faster intracellular build-up of PpIX and the drastically reduced concentration relative to ALA enables treatments with significantly lower doses and shorter application times. Therefore, a significant decrease in costs should be associated with the use of such esters. Faster production of PpIX and hence shorter instillation times may play an important role for commercialization of this technique. Moreover, the enhancement of lipophilicity, which has been achieved by esterification, will result in deeper penetration of the drug into targeted lesions after topical application and possibly also in a more homogeneous distribution of the resulting photosensitizer. Therefore, more efficient PDT mediated by such prodrugs will be possible. No direct relationship between lipophilicity and total PpIX build-up has been found, indicating that two different processes, uptake and ester cleavage, are necessary for efficient PpIX formation. Moreover, long-chained esters should always be applied with lower doses than ALA. In most cell lines h-ALA has shown the most efficient PpIX formation.

A further enhancement of PpIX formation can be obtained by an adjustment of applied ALA and ALA prodrug formulations to physiological pH values.

Acknowledgements

The authors would like to thank John-David Aubert (Division of Pulmonology, CHUV Hospital) for providing the BEAS-2B cell cultures and Pierrette Dessous l'église Mange (Institute of Pathology, CHUV Hospital) for her assistance in maintaining the different cell lines. We gratefully thank Yann Berger and Reinhardt Neier (Institute of Chemistry, University of Neuchâtel) for the synthesis of ALA cyclohexyl ester. This work was supported in part by the Swiss 'Fonds National' (Grant 20-50691.97), the Swiss National Priority Program in Optics, the Swiss Cancer Foundation, the Swiss Society for Multiple Sclerosis, and the 'Fonds Vaud–Geneva' for their financial support. N.L. would like to thank the Schering Research Foundation for providing his grant.

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PPIX production measured by fluorescence spectroscopy in Vitro for different precursors

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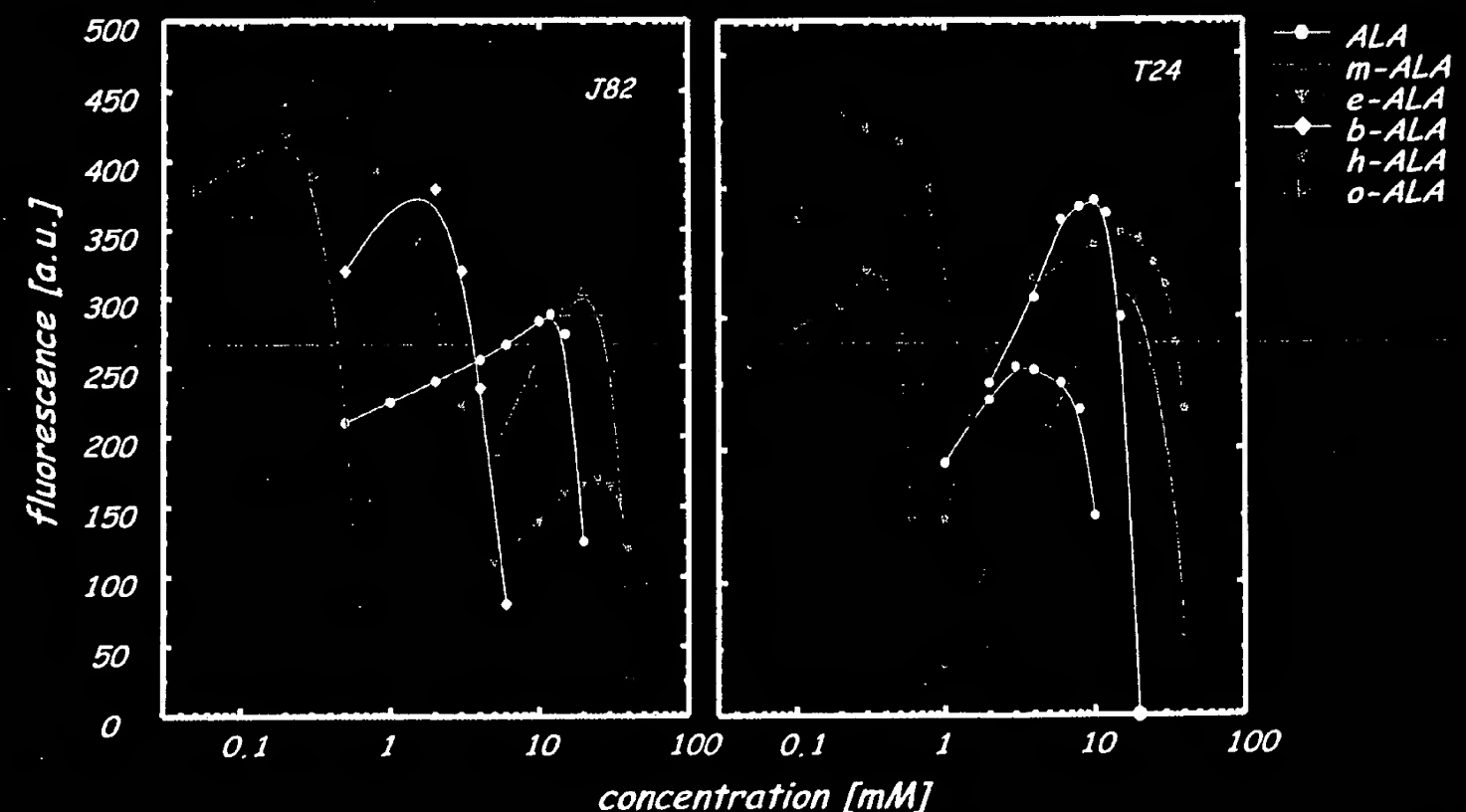

0022-5347/99/1615-0001/0
THE JOURNAL OF UROLOGY
Vol. 161, ●●●-●●●, May 1999
Printed in U.S.A.

OPTIMISATION OF THE FORMATION AND DISTRIBUTION OF PROTOPORPHYRIN IX IN THE UROTHELIUM: AN IN VITRO APPROACH

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ABSTRACT

Purpose: To optimize conditions for photodynamic detection (PDD) and photodynamic therapy (PDT) of bladder carcinoma, urothelial accumulation of protoporphyrin IX (PpIX) and conditions leading to cell photodestruction were studied.

Materials and Methods: Porcine and human bladder mucosae were superfused with derivatives of 5-aminolevulinic acid (ALA). PpIX accumulation and distribution across the mucosa was studied by microspectrofluorometry. Cell viability and structural integrity were assessed by using vital dyes and microscopy.

Results: ALA esters, especially hexyl-ALA, accelerated and regularized urothelial PpIX accumulation and allowed for necrosis upon illumination.

Conclusions: hexyl-ALA used at micromolar concentrations is the most efficient PpIX precursor for PDD and PDT.

KEY WORDS: aminolevulinic acid, photodynamic detection, photodynamic therapy, urinary bladder, cancer, in vitro

REFERENCE 1

Urinary bladder tumors show an increasing incidence in man after the sixth decade. They consist mainly of superficial transitional carcinomas and are characterized by frequent recurrence and/or risk to progress toward invasive tumors.[1] This is linked to their frequent multifocal character and concomitant presence of high grade dysplasia (DYS) centers and/or carcinomas in situ (CIS).[2]

The treatment of superficial bladder tumors is based mainly on endoscopic resections combined with chemo- or immunotherapy by intravesical installation. While the use of BCG is likely to modify the recurrence profile of the illness, reduce the risk of progression and improve the survival,[3] the resistance to BCG of certain tumoral bladders and decrease of vesical compliance resulting from repetitive treatments remain therapeutic problems.

As an alternative, photodynamic therapy (PDT) aims at destroying malignant cells by inducing cytotoxic reactions which result from interaction of light with photosensitive endo- or exogenous compounds, often preferentially accumulating in the target tissues. This concept led to development of several oncological treatments, for example, in dermatology, otorhinolaryngology, gastrology, ophthalmology and gynaecology.

In urology, where the main indication for PDT is multirecurrent superficial bladder cancer resistant to BCG treatment, PDT has received only marginal interest because the first generation photosensitizers did not localize with sufficient selectivity in neoplastic tissues and induced skin photosensitivity after systemic administration. Recently, interest in PDT of bladder cancers has been renewed by demonstration of the selectivity of protoporphyrin IX (PpIX) induced after instillation of 5-aminolevulinic acid (ALA). PpIX is an intermediate of the cycle of heme synthesis (fig. 1) and its intracellular content can be significantly increased when the regulatory step of the cycle is bypassed by exposing the tissue to a precursor, for instance 5-aminolevulinic acid (ALA).[4] In addition, Pp IX accumulates at much higher concentrations in malignant than in normal cells due to the reduction of ferrochelatase and iron deficiency in tumors.[5]

The results obtained by PDT in skin tumors[6] suggested that a similar approach might be used in urology. While the diagnosis of CIS and DYS is difficult or impossible during cystoscopy using white light, fluorescence cystoscopy after intravesical administration of 3% ALA solution often allows us to detect and define with precision the limits of DYS and CIS.[7,8] The sensitivity and specificity of photodynamic detection (PDD) approach 80%. The preferential accumulation of PpIX in the transformed urothelium,[9] the intravesical tolerance of ALA solutions adjusted to physiological pH values, and the absence of systemic effects reinforce the interest of such an approach.

A complete destruction of a tumor by PDT critically depends on a sufficiently high concentration and homogeneous distribution of PpIX in the malignant cell layers.[10] Although relatively high ALA concentrations were instilled into the bladder for many hours, fluorescence microscopy showed a rather irregular distribution of PpIX within superficial tumors of the bladder.[11] Also, the conditions for reaching the threshold of phototoxicity in the urothelium are not exactly known. This is not surprising as a double charged molecule like ALA is not expected to penetrate with ease across cell membranes and interstitial spaces. More lipophilic derivatives of ALA are expected to be more favored from this point of view. After traversing the cellular membrane non-specific esterases will reduce such compounds to 5-ALA. Dimethylsulfoxide (DMSO) and desferrioxamine (DES) have been

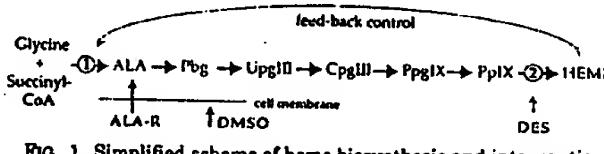

FIG. 1. Simplified scheme of heme biosynthesis and interventions used. ALA: 5-aminolevulinic acid; ALA-R: esters of ALA; Pbg: porphobilinogen; UpgIII: uroporphyrinogenIII; CpgIII: coproporphyrinogenIII; PpgIX: protoporphyrinogen IX; PpIX: protoporphyrin IX. 1: ALA synthase; 2: ferrochelatase + Fe++. DMSO: dimethylsulfoxide; DES: desferrioxamine. Gray arrows: inhibitory effects.

Accepted for publication November 8, 1998.
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Supported by grant 17 from the Geneva-Vaud Foundation and the Deutsche Forschungsgemeinschaft (DFG), Bonn, Germany.

and to enhance PpIX accumulation. DMSO increases the transmembrane passage of small molecules.[12] DES, chelates the intracellular iron and hence inhibits the ferrochelatase activity. Consequently, PpIX is not converted into heme (fig. 1) and accumulates in cells.[5]

Thus, to define standardized and optimal conditions for PDD and PDT, a systematic study of the penetration of ALA derivatives into cells, the kinetics of PpIX accumulation, intra-urothelial PpIX distribution and mechanisms of cell photodestruction is mandatory. As a first approach to this problem, we developed an experimental setup allowing us to answer some of these questions by using bladder mucosae explanted in vitro. Here we present the kinetics and tissue profiles of intracellular PpIX accumulation, and data about phototoxicity obtained in pig or human urothelium exposed to ALA, its esters, and ALA with DMSO or DES.

MATERIALS AND METHODS

Preparation of bladder mucosa. The study required living urothelium obtained under controlled conditions. Porcine urothelium resembles human urothelium structurally[13] and can be obtained easily and reproducibly. Porcine bladders were excised from slaughtered animals. Pieces of human bladder wall were obtained from patients undergoing radical cystectomy for advanced carcinoma (3 males, 1 female; average age 73 ± 6 years). Resected bladders were opened and de visu normal and flat areas were taken for experiments. All these manipulations took about 45 minutes. Normality was confirmed by histology. The protocols were approved by the state commissions controlling animal experiments and clinical research.

The tissues were stored at 4C in Tyrode solution. The urothelium was microdissected from the bladder wall using fine scissors. The plane of cleavage passed as near as possible to the basal membrane so that thin sheets of urothelium with remnants of lamina propria connective tissue were obtained.

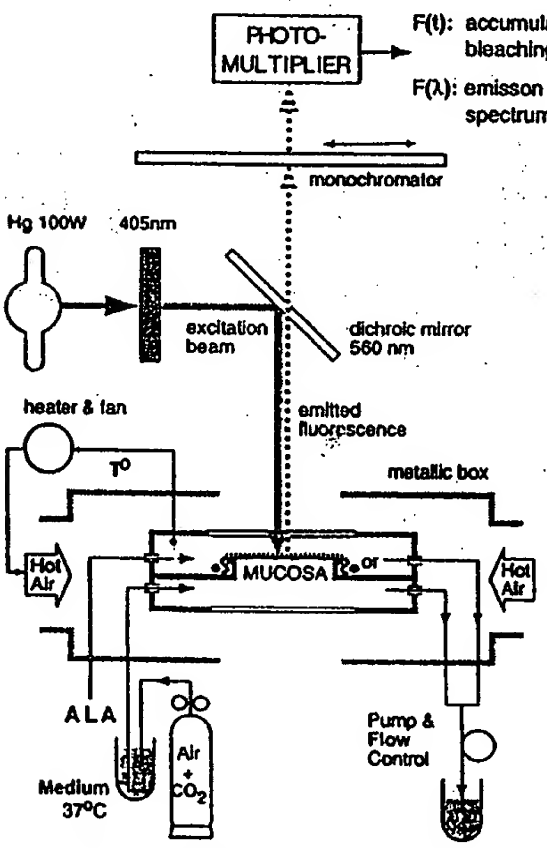

FIG. 2. Microspectrophotometry of protoporphyrin IX in bladder mucosa. Bladder mucosa is placed over and around circular rim of perspex plate and fixed with thin silicone O-ring (or). Preparation is mounted into transparent chamber and incubated in presence of ALA derivatives. Mucosa is periodically excited (100W mercury lamp, Eppendorf filter 405 nm (FWHM: 12 nm), 200 msec exposure, 45 ± 5 μW/0.05 mm.[2]) and fluorescence emitted by cells is recorded by using EMI 20 photomultiplier. Motorized monochromator (continuous interference filter Veril, Leitz) allows analysis of emission spectrum.

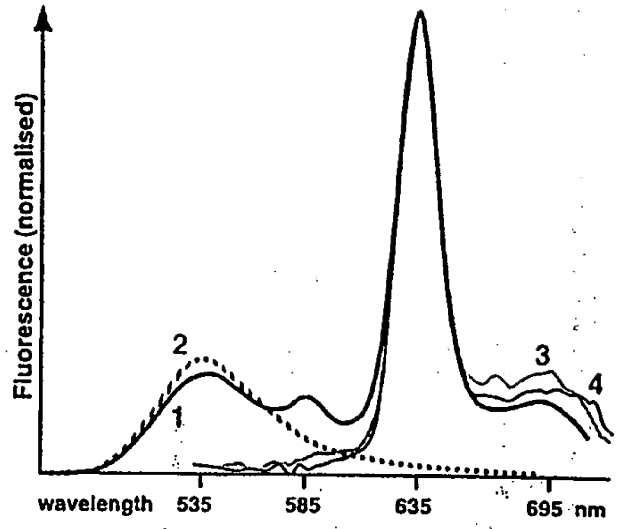

FIG. 3. Specificity of fluorescence signal. Four emission spectra as obtained from living mucosae and from frozen sections of urothelium. 1, living mucosa preincubated for 2 hours with ALA. 2, same mucosa after 10 minutes illumination. 3 and 4, urothelial sections incubated with ALA+DES and H-ALA, respectively. Spectra 1, 3 and 4 show identical peaks around 635 and 690 nm corresponding to PpIX accumulated in urothelium. In spectrum 2, PpIX signals disappeared leaving only tissue autofluorescence. Note that emission peak at 670 nm of curve 3 is due to photooxidation products of PpIX.

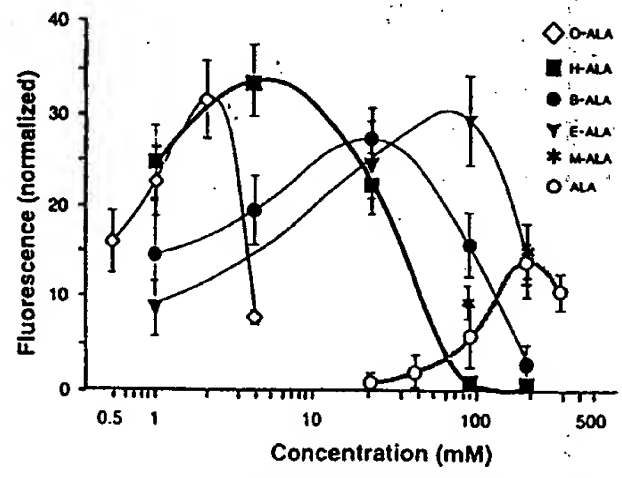

FIG. 4. PpIX kinetics for different precursors. O-ALA and H-ALA are most efficient precursors tested: at concentration 100 times lower than that of ALA, they induce 2 to 3 times higher PpIX accumulation. Determinations are made after 1 hour of incubation. Values are means ± S.D. from 20 measurements (4 mucosae per condition, 5 measurements in each mucosa). Values for M-ALA, determined at 90 and 180 mM only, were 9.4 ± 1.8 and 15 ± 3 respectively.

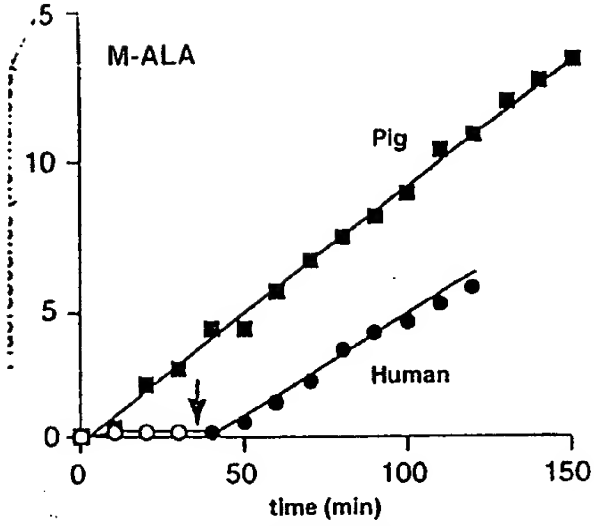

FIG. 5. Comparison of PpIX fluorescence in pig and human mucosae. During first 3 hours of incubation fluorescence increase is nearly linear in pig mucosa. Pig mucosa accumulates PpIX faster ($F = 0.082$ min., $r^2 = 0.99$) than human mucosa ($F = 0.062$ min., $r^2 = 0.98$). PpIX fluorescence is only observed when human mucosa is warmed up. M-ALA was administered at time 0. Arrow: warming of human mucosa; empty circles: human mucosa at 23C; full symbols: mucosae at 36C.

These were cut into 7 × 7 mm. fragments which were mounted (urothelium up) in a transparent culture chamber designed for epithelia[14] as illustrated in fig. 1. The mucosa divided the chamber into superior and inferior compartments (diameter 20 mm., height 3 mm.) and the area exposed to exchanges was 0.125 cm.^{2} The chamber was fixed onto the plate of an epi-illumination microscope (Leitz Orthoplan) and thermostabilised at 36 ± 0.5C. The inferior compartment was continuously perfused by oxygenated Tyrode solution. Solutions of ALA derivatives were injected as a single dose into the superior compartment.

Media. The Tyrode solution contained (in mmol/l.): 143.0 Na^+, 2.0 K^+, 0.8 Mg^{++}, 1.4 Ca^{++}, 122 Cl^-, 20.0 HCO_3^-, 3 $H_2PO_4^-$, 1.2 SO_4^{--}, 8 glucose (osmolarity 290 mOsm/l.) and was saturated with air enriched with 5% CO_2 (pH 7.5). ALA and some of its derivatives were dissolved in phosphate buffer saline at 4C, and the pH was adjusted to 5.2 ± 0.5. Dimethylsulfoxide (DMSO) (17.5 μM) and desferrioxamine mesylate (DES) (15 μM) were added to some solutions. All solutions were colorless. They were stored on ice and used within one hour.

ALA was from Merck (Dietikon, Switzerland), methyl-ester (M.-ALA) and DES were from Fluka (Buchs, Switzerland). Ethyl-(E-ALA), butyl-(B-ALA) hexyl-ester (H-ALA) and octyl-ester (O-ALA) were synthesized.[15] Their purity was superior to 95%.

Spectrofluorometry. The kinetics of urothelial PpIX accumulation with respect to precursor concentration and to time of administration were characterized as follows (fig. 1). The urothelium, incubated with a given precursor, was excited by violet light (405 nm, 45 ± 5 μW/0.05 mm.^{2}, 200 ms) each 10 minutes or each hour and the fluorescence emitted by the cells, which is taken to be proportional to the cell PpIX concentration, was passed through a low pass filter (>610 nm) and recorded by a photomultiplier. The specificity of the fluorescence signal was systematically checked by analyzing the emission spectra.

The spatial distribution of PpIX across the mucosa was determined at selected time intervals in serial 25 μm. thick frozen sections. To avoid strong photobleaching due to light exposure, the samples were prepared in the dark. The profiles of PpIX fluorescence within the mucosa were determined by scanning the fluorescence signal across the section.

Cell viability. At the end of experiments, the urothelium was exposed to acridine orange (dissolved in Tyrode 1:10000) which stains nuclei of living cells only. The proportion of labeled nuclei was evaluated by fluorescence microscopy (excitation at 405 nm, emission> 560 nm). In some cases, the time-course of PpIX photodestruction (photobleaching) was determined and the consequent phototoxicity effects on urothelial cell were studied by using electron microscopy. Two hours after the exposure to light, the mucosae were fixed in paraformaldehyde/glutaraldehyde and embedded in Epon. Sections of 700 Å were analyzed by transmission microscopy (Zeiss, Germany). Some mucosae were dehydrated and dried (CPD 030 critical point dryer, Balzers, Liechtenstein), coated with 300 nm gold (S150 sputter coater, Edwards, Zivy, Basle) and studied by using scanning electron microscope (JEOL, Tokyo).

Statistical analysis. Supposing that the photobleaching of endogeneous chromophores is small, fluorescence values were normalized, that is, corrected for the tissue autofluorescence [$I_n(t) = (I_n(t)\text{-}AF)/AF$)]. The data are presented as arithmetical means and standard deviations. A paired bilateral t test was used to compare the results and values of $p \leq 0.05$ were considered as significant.

RESULTS

Nature of the fluorescence signal. The spectral analysis of the light emitted by the mucosa shows that, in the absence of PpIX precursors, the tissue emits weak autofluorescence giving a very small signal in the red domain (≥610 nm). This signal serves to normalize the specific PpIX fluorescence. The latter appears in presence of PpIX precursors as one major

Pp IX accumulation in urothelium in response to ALA derivatives

Precursor	Hours of Administration						
	1	2	3	4	5	6	7
ALA	17 ± 7	31 ± 7	41 ± 6	64 ± 9	64 ± 7	67 ± 10	65 ± 12
ALA+DMSO	14 ± 3	36 ± 8	57 ± 17	75 ± 17	90 ± 18	90 ± 18	87 ± 31
M-ALA	15 ± 3	35 ± 5	53 ± 5	69 ± 9	82 ± 13	94 ± 14	91 ± 20
M-ALA+DMSO	18 ± 3	42 ± 5	63 ± 9	79 ± 11	97 ± 14	115 ± 13	114 ± 17
ALA+DES	21 ± 2	48 ± 11	81 ± 13	117 ± 14	157 ± 36	193 ± 50	256 ± 27
B-ALA	27 ± 3	61 ± 7	100 ± 14	134 ± 26	163 ± 28	156 ± 27	144 ± 25
H-ALA	34 ± 4	67 ± 6	105 ± 12	134 ± 20	167 ± 27	175 ± 26	189 ± 37
H-ALA+DES	32 ± 3	62 ± 5	98 ± 4	126 ± 10	153 ± 7	172 ± 8	170 ± 16

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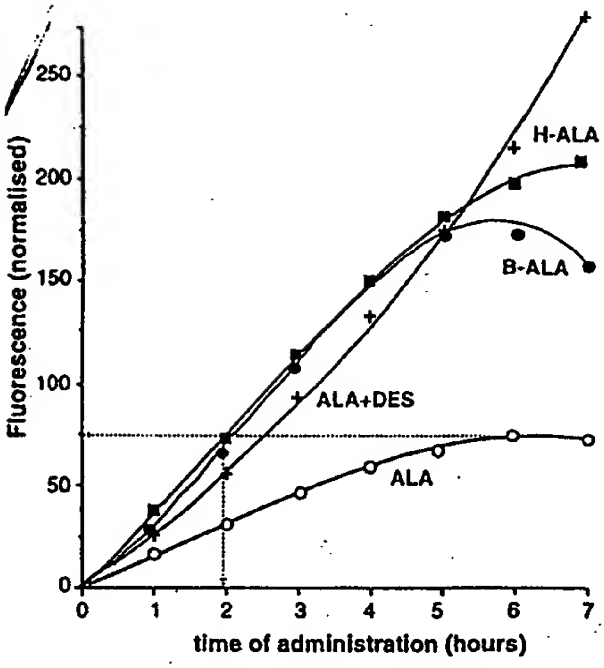

FIG. 6. PpIX accumulation with ALA, ALA+DES, B-ALA and H-ALA. H-ALA, although administered at much lower, and hence less toxic concentration, can considerably shorten time of administration (dotted lines), allowing both rapid and efficient PDD and PDT. (ALA and ALA+DES: 700 mOsm; H-ALA: 290 mOsm). Values are means extracted from table.

(635 nm) and one minor (690 nm) peak (fig. 3). No differences were observed between the emission spectra shape of pure ALA- and ALA-esters-induced PpIX. The PpIX peaks disappear after exposure of the mucosa to light.

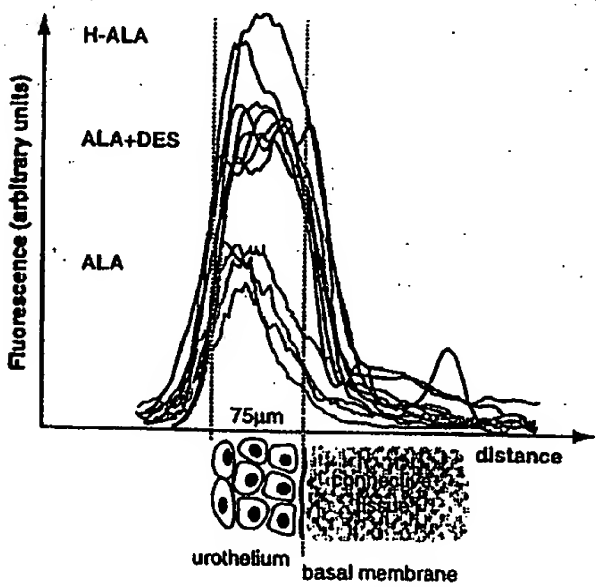

FIG. 7. Fluorescence intensity as measured across bladder mucosa. H-ALA allows highest and most homogeneously distributed PpIX accumulation in urothelium. Data from 12 fluorescence scans across mucosal sections were corrected to mean urothelial thickness. Scanning speed 100 mm/sec.; excitation at 405 mm.; width of illuminated slit 30 µm.; emission at 610 nm).

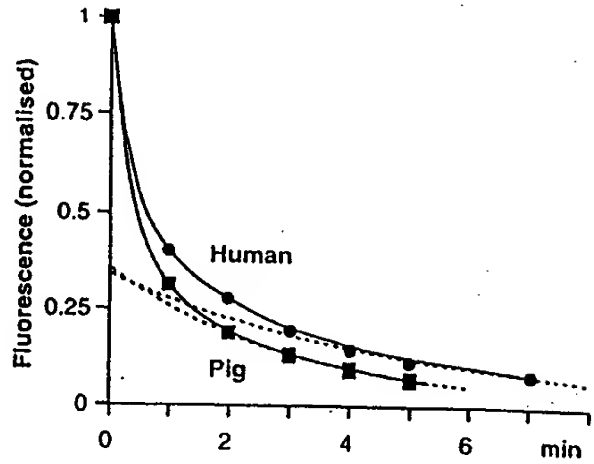

FIG. 8. Photosensitivity of fluorescence signal. Time-course of photodestruction of PpIX upon continuous illumination (405 nm, 4.75 J/cm^2) of mucosa. Photobleaching appears to show fast and slow phases. Residual signal corresponds to autofluorescence.

Effect of PpIX precursor concentration. Fig. 4 illustrates the results obtained for ALA and its esters after 1 hour of incubation. All precursors show similar kinetics: with increasing concentration, the fluorescence intensity increases, reaches a maximum and then decreases sharply. As compared with ALA, the applied concentrations of E-ALA, B-ALA, H-ALA and O-ALA are respectively 2, 10, 45 and 90 times lower, but nevertheless result in 2 to 2,5 times higher fluorescence. F4

The reduction of fluorescence at high concentrations may indicate significant cytotoxicity of ALA esters which resulted in peeling of the mucosa (as with ALA at 180 mM), or even immediate cellular lysis (as with H- and B-ALA at 180 mM)(not shown). Due to its higher lipophilicity, precipitation of O-ALA in aqueous solutions at high concentrations may reduce the total drug content.

Accumulation of PpIX in the mucosa. Both the pig and human mucosae exposed to precursors accumulate PpIX, after warming to 37C, that is, upon metabolic activation. This is illustrated in fig. 5 which shows an example of human mucosa with an accumulation ratio comparable to that of pig mucosa. However, on the average, the human mucosae (n = 4, 3 males, 1 female, mean age 52 years) accumulated PpIX 3.6 times less than the pig mucosae. F5

The table shows the results obtained in the pig mucosae with ALA derivatives used at their respective optimal concentrations. In all cases, the fluorescence increased nearly linearly up to four hours and saturated between the 6th and 7th hours. With ALA+DES, the fluorescence continued to increase exponentially up to 7 hours. ALA was the least efficient of the tested precursors. The other substances induced a significantly faster and greater (1.3 to 3 times) increase of PpIX fluorescence. H-ALA and ALA+DES were the most efficient, but H-ALA and B-ALA were shown to reach the highest fluorescence at the shortest administration times (table, fig. 6). No significant difference in PpIX formation was observed between H-ALA and B-ALA used at their optimal concentrations. This indicates that the biosynthetic pathway of heme was saturated by the more lipophilic esters, while PpIX production induced by pure ALA never reached sufficiently high intracellular drug contents. T1 F6

Iso-osmolar replacement of sodium in the Tyrode solution by choline did not modify the accumulation kinetics of PpIX (not shown) indicating that the penetration of ALA derivatives into the cell does not involve a sodium-dependent co-transport.

At the end of each experiment (24 mucosae, 7 hours of

fication, 8 precursors at their optimum concentration), an labeling with acridine orange, all preparations showed lls with bright nuclear fluorescence, indicating that the othelium remained alive.

Distribution of PpIX across the mucosa. The fluorescence ofiles across the mucosae were recorded after 2 hours of ubation with ALA, H-ALA and ALA+DES. As the thickss of urothelium varied (from 66 to 88 μm.), the results are esentod after a homothetic translation to 75 μm. As shown fig. 7, PpIX fluorescence induced by the 3 precursors is nited essentially to the urothelial cells. With ALA, the orescence is limited mostly to the superficial cells while th ALA+DES and especially H-ALA, the fluorescence is out twice as high and distributed in all urothelial layers.

Phototoxicity. If the mucosa accumulating PpIX is exposed continuous violet illumination for 10 minutes, the urothed fluorescence decays. Supposing an exponential decay th time, the two rate constants are of about 30 seconds and 3 inutes (fig. 8). This time dependence of the fluorescence signal ay be due to more stable photoprotoporphyrins formed by otodegradation of PpIX. After 10 minutes of illumination, the specific fluorescence is no longer detectable which indicates that most fluorescing porphyrins were destroyed.

When the mucosae illuminated for 10 minutes were incubated for 2 additional hours, the cells that had been exposed to light died. This was documented by electron microscopy (fig. 9) which revealed damaged mitochondria, marginalisation of nuclear chromatin, vacuolised cytosol and fenestration of the plasma membrane. The superficial cells were rounded and lost contact with each other. In mucosae preincubated for shorter times (for example, ALA, 2 hours), the necrotic changes were found mostly the in the superficial cells. In mucosae preincubated for longer times (for example, ALA, 6 hours) the urothelial necrosis was complete while the underlying connective tissue was not damaged. The necrosis induced by violet light was confined to the illuminated area and was surrounded by normal cells (fig. 9). F9

DISCUSSION

The use of bladder mucosa explanted into a superfusion chamber is a powerful tool which, unlike cell cultures, per-

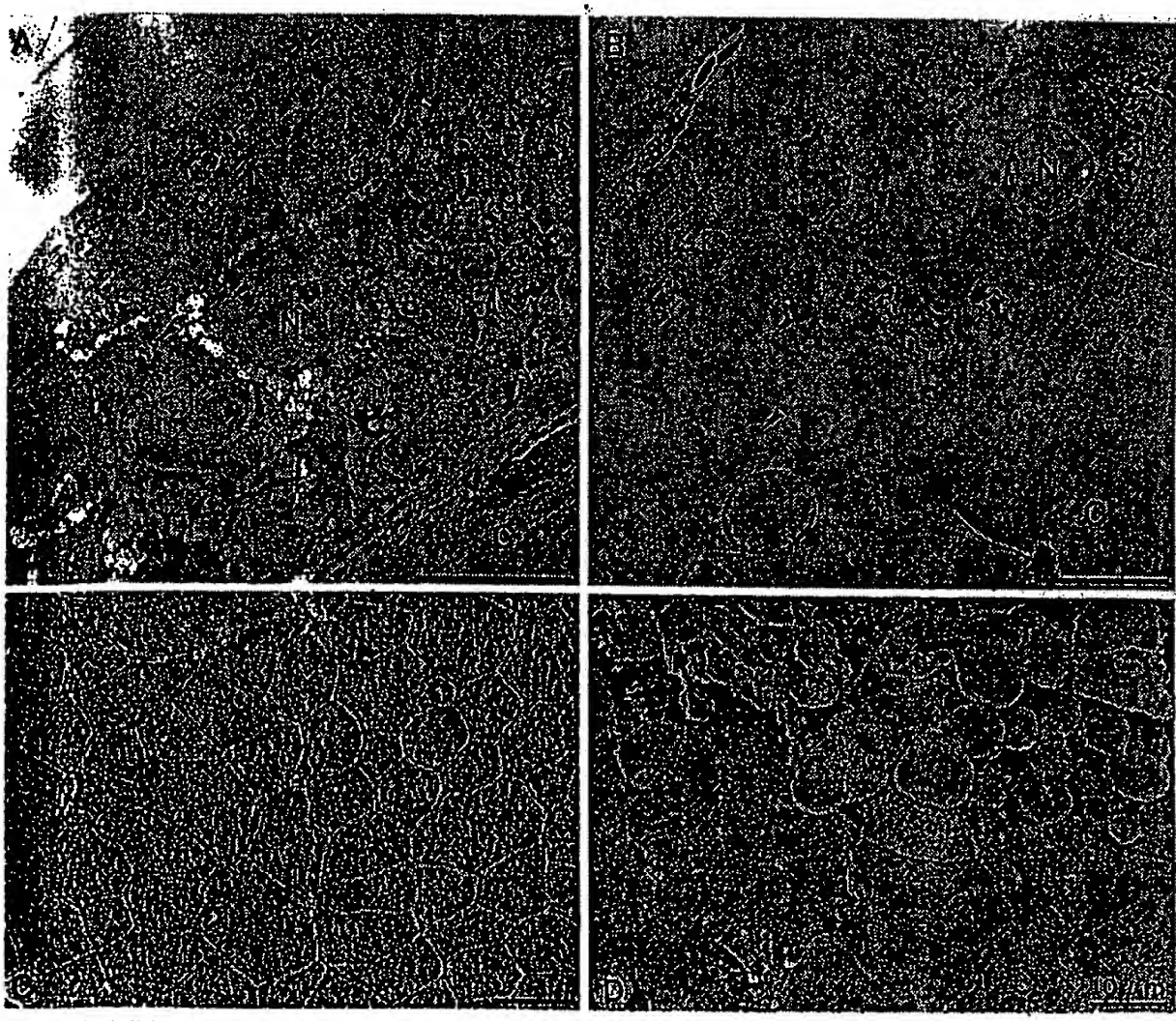

FIG. 9. Urothelial necrosis induced in bladder mucosae by exposure to light. Transmission (*A, B*) and scanning (*C, D*) electron micrographs of g mucosae incubated for 6 hours with ALA. *A, C*, control mucosae with normal intra- and intercellular structure. *B, D*, mucosae exposed to light 05 nm, 4.75 J/cm.^{2}, 10 minutes) and incubated for 2 additional hours, showing marginalized chromatin, swollen mitochondria, vacuolized toplasm, fenestrated plasma membrane and lost intercellular contacts. Arrows: mitochondria, N: nuclei, C: connective tissue. Bars: 10 μm.

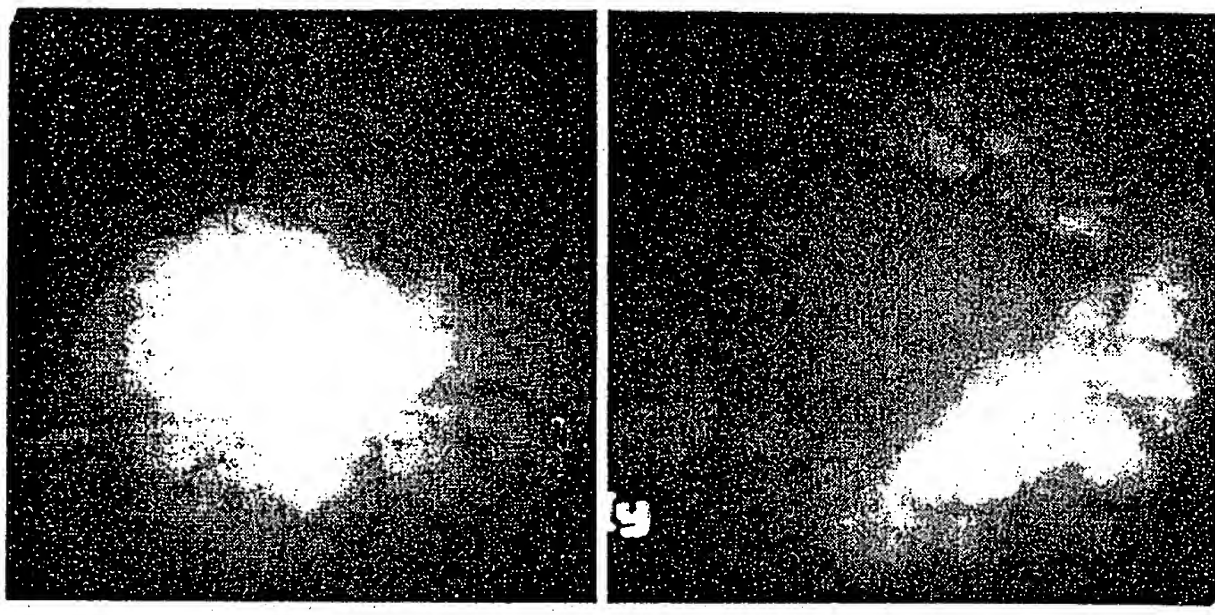

FIG. 10. Endoscopic view of human bladder papilloma. Left: image in white light. Dimension of tumor is 4 × 8 mm. Right: image in ue-violet light after instillation of hexyl-ALA (8 mM) for 2 hours. Highly fluorescent tumoral cells (red) appearing on background of n-fluorescent (green) normal tissue. Histological diagnosis: p'faG1. (With permission.[24])

its the study of epithelia with intact architecture and functional polarity. This is especially valuable for evaluation of enetration of substances into the normal as well as pathological epithelia. Although the blood circulation is eliminated this preparation, the renewal of submucosal medium sures the homeostasis and survival of urothelial cells.

Urothelium of pig and human origins has a very similar ructure although surface proteoglycans are not identical d mucus cells are absent in the normal human bladder.[13] our hands, both mucosae also show similar accumulation netics although the final fluorescence intensity in the human case is lower. This reflects possibly decreased cell viability due to unavoidable and long (about 2 hours) hypoxia sulting from early vascular ligation during the bladder erations.

The synthesis of supplemental PpIX must be preceded by enetration of the precursor across the plasma membrane. hree results strongly suggest that the precursors penetrate to the cells by simple diffusion:[1] esters with the longer liphatic moiety (hence more lipophilic) penetrate faster and a greater extent,[4] similar kinetics of PpIX formation for -ALA (4 mM) and B-ALA (20 mM) as well as comparable uorescence levels under optimized concentrations suggesting a passive concentration gradient driven uptake as predicted by simple diffusion laws,[3] and absence of sodium in the olutions does not decrease the PpIX accumulation as would e expected for a sodium-linked co-transport frequently operating for amino-acid cell transport. This is in agreement ith results obtained in cell cultures.[16]

Once inside the cell, the esters of the ALA are hydrolyzed y non-specific cell esterases and free ALA appears in the ytoplasm.[16] The cell fluorescence will, however, increase ely upon metabolic activation in the mitochondria, which nfirms that PpIX synthesis is an energy-dependent process [2, 5].

Whatever the precursor used, the time profiles of accumulation are similar: with increased concentration, the synthesis of PpIX increases to a maximum and then decreases to ero. Similar results were obtained in cell cultures.[16] In our ase, the final decrease is accompanied by a loss of cell ohesion and presence of tiny cells floating in the superfusate. This might be due to the hypertonicity of the solutions[17] (fig. 4) and/or to the toxicity of ALA itself. Indeed, the cells also peel off in presence of diluted but highly penetrant precursors such as H-ALA where the resulting high cytoplasmic ALA concentration might favor the production of oxygen reactive radicals[18] and subsequent cell injury.

PpIX fluorescence increases essentially linearly up to 4 hours and then attains a plateau value or even slightly decreases. Similar results were found in cell cultures from tumoral bladders and in rat urothelium in vivo.[19, 24] It is possible that this plateau reflects not only the balance between PpIX synthesis and PpIX utilization, which should happen with all precursors, but also the penetration of precursors into deeper lying cells, which should increase with liposolubility of the ALA esters, and appears to be shown by the fluorescence profiles. The observed dependence of decreasing optimal concentration with increasing chain length of the ALA derivatives has been confirmed in cell cultures.[16] O-ALA at higher concentrations shows a tendency to precipitate at physiological pH values which limits its clinical use.

Clearly, DMSO, which is supposed to increase the membrane permeability, potentiates the PpIX accumulation under our conditions much less than the esterification of ALA. DES, a selective an iron chelator, when combined with ALA, inhibited the conversion of PpIX to heme and thus potentiated PpIX accumulation to higher levels and before a plateau was reached. This suggests that the synthesis of PpIX in itself was not slowed down in any of the conditions used. Surprisingly, DES showed no potentiation when combined with H-ALA. The explanation of this result awaits new experiments.

The photobleaching curves show a fast and a slow component, which might result from the bleaching of fluorescent photooxidation products, originating in different intracellular compartments.[24] A mixture of isomeric chlorins resulting from the first photodegradation process of PpIX has been shown to be about 10 times more stable to photo oxidation than PpIX.[24] Their appearance may be seen from the fluorescence emission peak at 670 nm (fig. 3, curve 3). Our results show that 10 minutes exposure to light is sufficient to induce cell necrosis in all layers of a normal urothelium. In

/o records of photobleaching could help to dose the radia-
n energy necessary to destroy the tumoral tissue.

CONCLUSIONS

The in vitro preparation of bladder mucosa developed in is work has brought additional valuable information on the namics of accumulation and destruction of photosensitive olecules used in the PDD and PDT of urothelial carcinoma. the case of PpIX, H-ALA seems to be a good compromise tween lipophilicity, solubility and performance with re-ect to high PpIX formation and low precursor concentra-n. In comparison with ALA, it increases and accelerates e PpIX synthesis, penetrates into all epithelial cell layers, d is efficient at low concentrations. At these low concen-ıtions it preserves urothelial viability and allows effective ll photodestruction. The optimal time necessary for PDD d PDT at the conditions applied is shortened from near 6 to ; hours. As shown in fig. 10, under these conditions applied humans, PDD results confirm the predictions and show lective accumulation of PpIX in urothelial carcinoma.

Acknowledgments. We thank C. Verdan for help with elec-ın micrographs and Dr. G. Wagnières for valuable discus-ıns.

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DOSE-RESPONSE OF DIFFERENT ALA-ESTERS ON THE PIG BLADDER MODEL

Marti et al., "Optimisation of the formation and distribution of Protoporphyrin IX in the urothelium: an in vitro approach", J. of Urology, 162(2), pp 546-552, 1999.

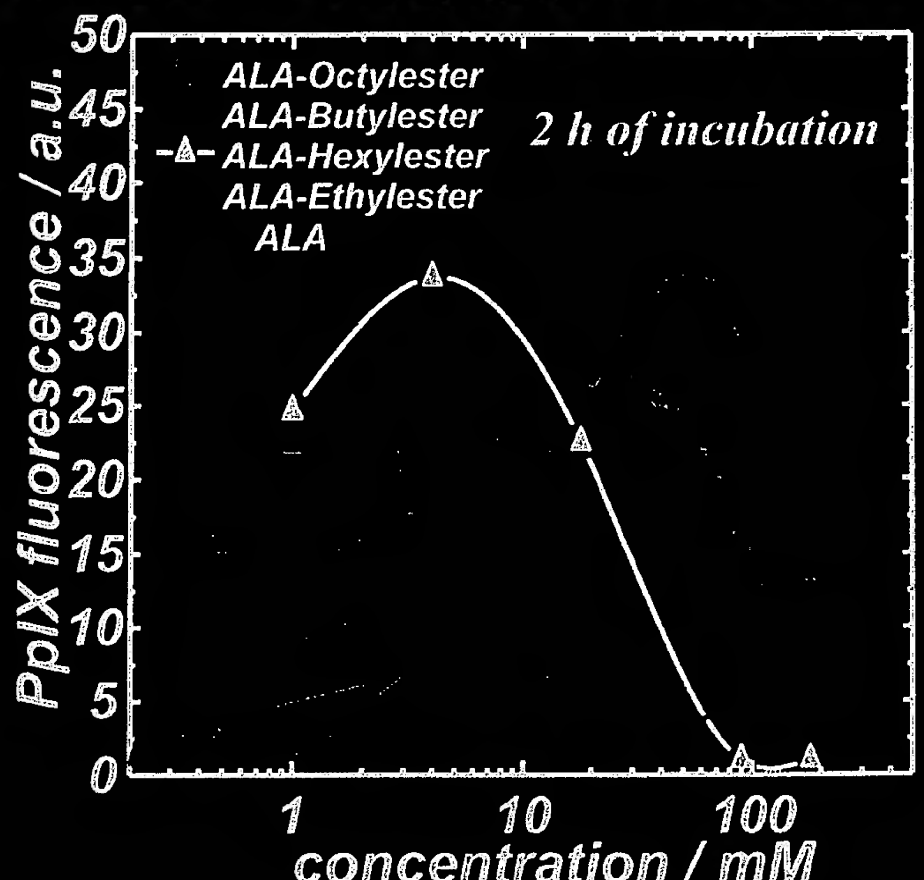

Table 1. Optimal Concentrations of ALA and ALA-esters for Protoporphyrin IX Formation.

Substance	Mw (HCL-salt)	Optimal concentration*		
		mM	mg/ml	% (w/w)
ALA	167.6	200	33.5	3.4
ALA ethylester	195.6	60	11.7	1.2
ALA butylester	223.8	20	4.5	0.4
ALA hexylester	251.8	4	1.0	0.1
ALA octylester	279.6	2	0.6	0.06

*Concentration resulting in optimal porphyrin formation

British Journal of Cancer (1999) 80(1/2), 185–193

Article no. bjoc.1998.0338

Photodetection of early human bladder cancer based on the fluorescence of 5-aminolaevulinic acid hexylester-induced protoporphyrin IX: a pilot study

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Summary Exogenous administration of 5-aminolaevulinic acid (ALA) is becoming widely used to enhance the endogenous synthesis of protoporphyrin IX (PpIX) in photodynamic therapy (PDT) and fluorescence photodetection (PD). Recently, results have shown that the chemical modification of ALA into its more lipophilic esters circumvents limitations of ALA-induced PpIX like shallow penetration depth into deep tissue layers and inhomogeneous biodistribution and enhances the total PpIX formation. The present clinical pilot study assesses the feasibility and the advantages of a topical ALA ester-based fluorescence photodetection in the human bladder. In this preliminary study 5-aminolaevulinic acid hexylester (h-ALA) solutions, containing concentrations ranging from 4 to 16 mM, were applied intravesically to 25 patients. Effects of time and drug dose on the resulting PpIX fluorescence level were determined in vivo with an optical fibre-based spectrofluorometer. Neither local nor systemic side-effects were observed for the applied conditions. All conditions used yielded a preferential PpIX accumulation in the neoplastic tissue. Our clinical investigations indicate that with h-ALA a twofold increase of PpIX fluorescence intensity can be observed using 20-fold lower concentrations as compared to ALA.

Keywords: 5-aminolaevulinic acid; 5-aminolaevulinic acid hexylester; photodynamic therapy; fluorescence; protoporphyrin IX; human bladder cancer

Fluorescence photodetection (PD) and photodynamic therapy (PDT) are techniques currently under clinical assessment for both visualization and local destruction of malignant tumours and premalignant lesions. One drawback of these methods found with some photosensitizers is a more or less long-term cutaneous photosensitivity (Wagnières et al, 1998; Dougherty et al, 1990). A more recent strategy for administering photosensitizers involves the application of 5-aminolaevulinic acid (ALA) in order to stimulate the formation of protoporphyrin IX (PpIX) in situ. The exogenous ALA bypasses the negative feedback control from haem to ALA synthase that catalyses the condensation of glycine and succinyl–coenzyme A (CoA). Given in excess, exogenous ALA thus can result in a temporary accumulation of PpIX, in particular, in cells with higher metabolic turnover. Since PPIX has fairly good photosensitizing properties (Cox et al, 1982; Kennedy et al, 1990) proposed ALA as a possible photodynamic agent. Following this pioneering work, this treatment modality has been widely studied for various cancers (Kennedy et al, 1992; Peng et al, 1992; Svanberg et al, 1994).

As well as for the PDT of malignant or premalignant lesions, ALA-induced PpIX is now being used for the detection of such lesions. This technique has been shown to work, among other applications, in urology, where easy instillation in the bladder, combined with the fact that this organ is readily accessible endoscopically, makes it an ideal object. Alongside classical techniques such as cytology or white light examination, fluorescence PD by ALA-induced PpIX provides some advantages (Leveckis et al, 1994; Kriegmair et al, 1996; Jichlinski et al, 1997). This inspection modality allows an exact mapping which pinpoints, with a high level of sensitivity and specificity, the locations of carcinoma in situ (CIS) as well as early stages of cancer-like dysplasias, which are normally difficult to recognize under white light examination.

However, when using topically instilled ALA for the PDT of CIS and precancerous lesions, this modality appears to be limited by the amount of ALA that enter the target cells or by the tissue penetration and the distribution of the resulting PpIX in the targeted tissue. Almost all of these possible disadvantages accompanying the use of ALA can be ascribed to the physical–chemical properties of the molecule itself. Applied under physiological conditions, ALA is a zwitterion (Novo et al, 1996). Because the lipid bilayer of biological membranes is relatively impermeable to charged molecules, the cellular uptake of ALA is shallow. Consequently, in order to increase the transport across cellular membranes, fairly high drug doses and increased administration times have to be used. This deficiency results in a low penetration depth (Warloe et al, 1992; Loh et al, 1993; Peng et al, 1995) and an ALA-induced PpIX distribution, which is not optimized for the PDT of the deep layers of nodular lesions in the urothelium (Iinuma et al, 1995; Chang et al, 1996) after topical ALA application.

Systematic studies have shown that the modification of a drug to an ester, an amide or a urethane by the addition of a long-chain hydrocarbon improves penetration through biological barriers (Bridges et al, 1979; Jain, 1987*a*, 1987*b*). After penetration into the cell, the ester derivative can then, for example, be hydrolysed back to the free ALA by non-specific esterases. Recently,

Received 24 July 1998
Revised 8 October 1998
Accepted 15 October 1998

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promising results were obtained with different alkylesters of ALA in vivo and in vitro (Kloek et al, 1996; Peng et al, 1996; Gaullier et al, 1997; Marti et al, 1998). These groups demonstrated that the application of esterified ALA derivatives results in an up to 25-fold increase in PpIX fluorescence levels as compared to ALA.

This report covers initial clinical investigations with 5-aminolaevulinic acid hexylester hydrochloride (h-ALA)-induced fluorescence PD in the human bladder. Following our preclinical studies (Marti et al, 1999), we selected h-ALA from the multitude of possible ALA-alkylesters because it represents a good compromise between water–urine solubility and sufficient PpIX formation capacity at low doses. Furthermore, h-ALA has been shown to lead to a homogenous distribution of PpIX-related fluorescence over the entire urothelium in our pig bladder model (Marti et al, 1998). In addition, it can be synthesized simply from ALA and hexanol (Kloek et al, 1997). The goal of this clinical pilot study was to test h-ALA as a potential candidate for improving both the PD and PDT in the urinary bladder. Therefore, topical application of h-ALA should result in higher PpIX formation than is the case with the same amount of ALA. It should enable shorter times between instillation and examination and lower drug concentrations while retaining the outstanding selectivity of ALA. This work presents a preliminary optimization of h-ALA-induced PpIX in respect to the resulting fluorescence intensities. Both the influence of the concentration and instillation time of h-ALA solutions on the total amount of PpIX were determined in vivo by the use of an optical fibre-based spectrofluorometer.

MATERIALS AND METHODS

Patients

Twenty-five patients (seven women and 18 men, four cases of ordinary ALA and 21 cases of h-ALA) have been involved in this first study conducted since August 1997. The mean age was 70 years, covering an age range of between 44 and 85. Local ethical committee approval was granted for this study, and written consent was obtained in each case.

Preparation and administration of ALA and h-ALA

ALA (99%) was purchased from Merck (Darmstadt, Germany). Other chemicals (thionyl chloride 99% and 1-hexanol 99.9%) used for the synthesis of h-ALA were ordered from Fluka Chemie AG (Buchs, Switzerland) and were used without further purification.

The synthesis described here is a slight modification of the methods reported recently (Takeya, 1992; Kloek et al, 1996). In brief, 3.5 ml of thionyl chloride were added drop by drop under stirring to an excess (~ 10 ml) of 1-hexanol cooled on ice in an

Table 1 Experimental instillation conditions used in the first clinical trials with h-ALA and normalized fluorescence levels on papillary tumours (pTa G2) obtained by normalization to reference cuvette

Patient no.	Concentration (mM)	Instillation time (h)	Resting time (h)	Fluorescence signal (r.u.)
1	4	2	–	16.2
2	4	2	–	11.2
3	4	4	–	34.5
4	4	4	–	20.5
5[b]	8	2	–	22.1
				38.4
6	8	2	–	36.2
7	8	2	–	46.7
8	8	2	–	–
9[b]	8	2	2	151.1
				102.0
10	8	2	2	115.8
11	8	2	2	147.5
12	8	4	–	66.4
13	8	4	–	72.6
14	8	4	–	63.4
15	8	4	–	73.8
16	8	4	–	94.4
17	8	4	–	77.1
18	8	4	2	102.7
19	8	4	2	95.0
20	16	2	–	15.8
21	16	2	–	16.7
22	180[a]	4	2	54.0
23	180[a]	4	2	43.6
24	180[a]	4	2	46.2
25	180[a]	4	2	45.3

[a]Instillation of the 180 mM solution of ALA.[b]Patient with two papillary tumours.

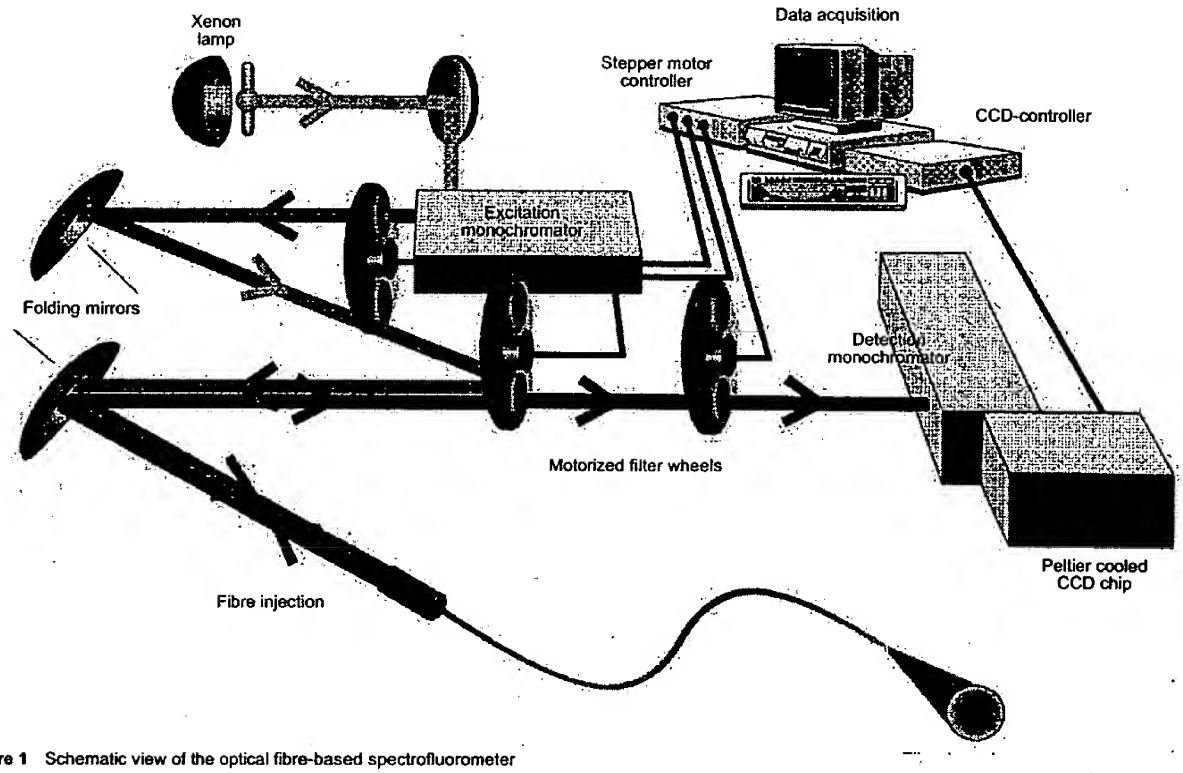

Figure 1 Schematic view of the optical fibre-based spectrofluorometer

argon atmosphere. The solution was stirred for a further 30 min to bring the reaction to completion; after warming up to room temperature, 2.5 g of ALA (M_r = 167.6 g mol^{-1}) were added to the solution. The suspension was then stirred overnight at room temperature under argon. The final phase of the reaction was controlled on-line by thin layer chromatography (TLC) (TLC foils; Schleicher & Schuell, Merck, Darmstadt, Germany) in CH_2Cl_2/MeOH (9:1) stained by $KMnO_4$ (Rf = 0.6). Once the reaction was complete, the solvent and hexylchloride were removed under reduced pressure (~ 0.5 torr). The viscous residue was dissolved in warm methanol. Then a small amount of methanol was evaporated until the first crystals of the reaction product appeared. A small quantity of diethylether was added and h-ALA was allowed to crystallize on ice. This dissolving and recrystallizing procedure was then subsequently repeated until only one spot was recognized on the TLC, yielding 80–90% of h-ALA (M_r = 251.8 g mol^{-1}) as a white powder. The product was characterized by proton nuclear magnetic resonance (^{1}H-NMR) with a 400 MHz (Bruker, Germany) spectrometer and identified as 5-aminolaevulinic acid hexylester hydrochloride. The purity (> 95%) was further verified by high performance liquid chromatography (HPLC) with UV/VIS detection at 270 and 350 nm respectively (data not shown). No other products were observed.

The ALA solutions were administered in accordance with standard protocol used in Lausanne's CHUV Hospital (Jichlinski et al, 1997). In brief, 1500 mg of ALA were dissolved in 38 ml of sterile water. Five millilitres of phosphate-buffered saline (PBS) were added and the pH was adjusted with a further 7 ml of aqueous sodium hydroxide (1N) to a value of pH 5.3. This solution with a concentration of 180 mM of ALA was sterilized by filtration through a Millipore filter (Millipore, Millex GS 0.22 μ) and stored at –18°C 1 day before measurements were conducted. The solution was instilled into patients' bladders using a 16 French Foley catheter 6 h prior to photodetection. Patients were asked to retain the solution for 4 h. Their bladders were evacuated 2 h prior to treatment.

Depending on the prodrug concentration to be applied, 50–200 mg (i.e. 4–16 mM) of crystalline h-ALA were dissolved in 35 ml of water. Then 13 ml of PBS were added to the aqueous solution and adjusted with 0.1 N hydrochloric acid to give the same pH value of 5.3. The solutions were instilled as described above. Table 1 summarizes the different conditions under which ALA and h-ALA were applied. All patients treated with ALA (four cases) and some instilled with h-ALA (five cases) had a supplementary resting time of 2 h after being exposed to the drug solution.

Procedure

Bladder inspection under white light illumination

Prior to further treatment or measurement, the actual status of the bladder was documented under white light illumination. The frame accumulation colour CCD camera (Storz, Tuttlingen, Germany), connected to a video recorder (JVC, Japan) and an RGB monitor (Sony, Japan) was plugged directly into the ocular of a 23.5 French cystoscope (Storz PDD, Tuttlingen, Germany) to record the standard endoscopic colour image.

Fluorescence spectroscopy

Fluorescence emission spectra were recorded with an optical fibre-based spectrofluorometer based on a Peltier-cooled CCD coupled to a spectrograph (Cromex 250, SI Instruments, Germany). The experimental setup is shown in Figure 1. Arranged on a trolley, the whole setup can be easily transported. Excitation light (λ_{ex} = 405 nm) from a 75 W high-pressure Xenon lamp (UXL-75 XE, Ushio Inc., Japan) was spectrally resolved by a quarter meter monochromator (Chromex 250, SI Instruments, Germany) with a bandwidth of 5 nm and an excitation filter, SCHOTT BG3 (Schott AG, Mainz, Germany), mounted on a filter wheel. A stepper motor (SMC 100, Princeton Instruments Inc., USA) controlled this excitation filter wheel, which was equipped with different low-pass filters installed to purify the excitation light. Fully reflective mirrors and a dichroic mirror (Reynard DC 450; Reynard, USA), mounted on a second filter wheel, were used to feed the light into a 600 µm core silicone-clad silica fibre with perpendicular polished end-faces. Excitation energy measured at the distal end of the fibre tip was determined with a calibrated power-meter (Optical Power Meter 840, Newport, USA). Fluorescence emitted by any sample was collected with the same fibre and separated from the excitation light by the dichroic optics described above. A long-pass filter (Reynard FG 455) mounted on a third filter wheel made further spectral separation, virtually eliminating all reflected excitation light prior to acquisition. This filter setup allows the acquisition of fluorescence emission spectra between 450 and 900 nm. Detection based on this combination enables fast data acquisition combined with a low level of noise. The whole setup and data acquisition was controlled by a 486 personal computer using CSMA software (SI Instruments GmbH, Germany).

An aqueous solution of Rhodamine B ($c = 1 \times 10^{-6}$ mol l^{-1}) in a 10 mm quartz cuvette was used as a reference. Emission spectra of the reference were recorded before and after each measurement. All measurements were normalized to the peak value of the reference to give comparable results corrected for day-to-day fluctuations of the excitation light energy or detection pathway alignment.

After inspection of the bladder under white light, the distal end of the fibre was introduced via the biopsy channel of the cystoscope. A background measurement was performed in the centre of the bladder to allow the correction of the spectra for parasitic light and fluorescence generated by the fibre itself. Then the physician brought the distal end of the fibre directly into contact with the bladder wall.

Bladder inspection under violet light illumination

After measurement of the fluorescence spectra (see below) of healthy, cancerous and suspicious areas in the bladder, the camera was equipped with a long-pass filter ($\lambda > 520$ nm; Wratten filter No. 12, Kodak, Rochester, USA), positioned between the ocular of the cystoscope and the CCD-Chip. A footswitch allows the physician to place a bandpass filter (380–450 nm) in front of the 300 W Xenon arc lamp (Storz, Tuttlingen, Germany) to give about 150 mW of violet light at the end of the cystoscope. Excitation with violet light generated a visible pale-green autofluorescence of the healthy mucosa. As a result of the absorption of autofluorescence, the blood vessels of the lamina propria appear somewhat darker. Filtration of the light below 520 nm allows these sites to be distinguishable from zones containing high PpIX concentrations, appearing in a clear, bright, fluorescing red. To improve the fluorescence images, the camera was switched into frame accumulation mode for enhanced sensitivity. The integration times ranged from one-eighth to one-half of a second, depending on observation distance.

Biopsy sampling and pathology

Prior to transurethral resection of the bladder wall (TURB), a total number of 109 biopsies from fluorescent and non-fluorescent areas (average 5.2 per patient; guided by light-induced fluorescence after excitation at 405 nm) were taken from the patients treated with h-ALA solutions. Macroscopic fluorescence findings and locations were documented for each biopsy. All samples were sent for histopathological examination. The urothelial carcinomas were graded and staged according to the World Health Organization (WHO) 1973 classification (Mostofio et al, 1973) and the UICC/AJC 1992 system (Hermanek and Subin 1992) respectively. Flat intra-epithelial neoplastic lesions were graded according to the criteria of Nagy et al (1982) and classified as grade 1 (mild dysplasia), grade 2 (moderate dyslasia), grade 3 (marked dysplasia) and carcinoma in situ.

RESULTS

Macroscopic findings

All aqueous solutions of h-ALA stayed clear and colourless until use. Neither systemic nor local reactions following the examination with both h-ALA and ALA were observed under the conditions used in this study. Even the highest drug dose administered (16 mM) of h-ALA was well tolerated. h-ALA-induced synthesis of PpIX was observed in each patient. All papillary and planar tumours, also visible under white light cystoscopy, showed bright red fluorescence. This red fluorescence was found to demarcate the outline of the urothelial lesions with high precision. Using the violet light of the filtered Xenon arc lamp, it was possible to perform both fluorescence-guided biopsies as well as accurate resections of targeted tissues. Qualitatively, all conditions tested resulted in a clearly visible contrast between healthy and diseased sites of the bladder wall.

Table 2 Correlation between histopathological finding and fluorescence diagnosis following h-ALA instillation

Histopathological findings	Total number of biopsies	Fluorescence positive	Fluorescence negative
Healthy mucosa	28	5	23
Metaplasia	1	1	–
Hyperplasia	3	3	–
Dysplasia G1	12	10	2
Dysplasia G2	5	3	2
Dysplasia G3	2	2	–
CIS	11	9	2
pTa G1	8	8	–
pTa G2	14	14	–
pTa G3	19	19	–
pT1 G2–G3	4	4	–
pT2a	2	2	–
Total	109	80	29

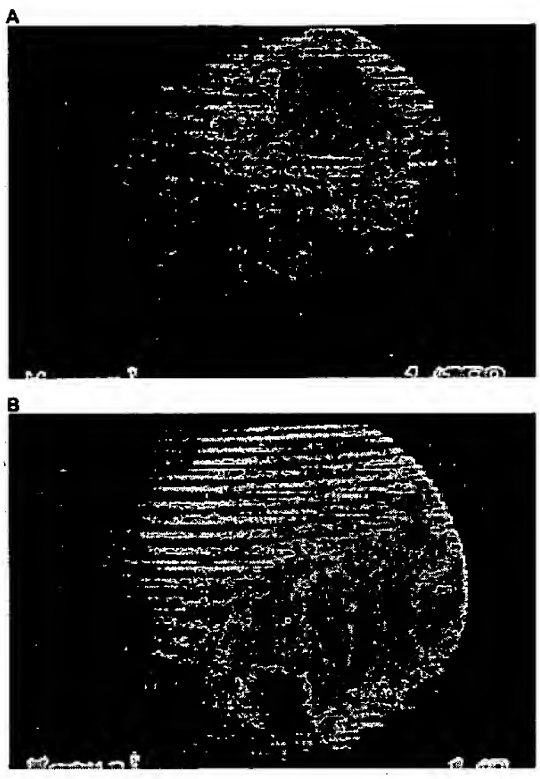

Figure 2 Endoscopic view of a flat papillary tumour (pTa G2) after 2 h of h-ALA exposure (for description see text) under (A) white light, (B) violet light examination

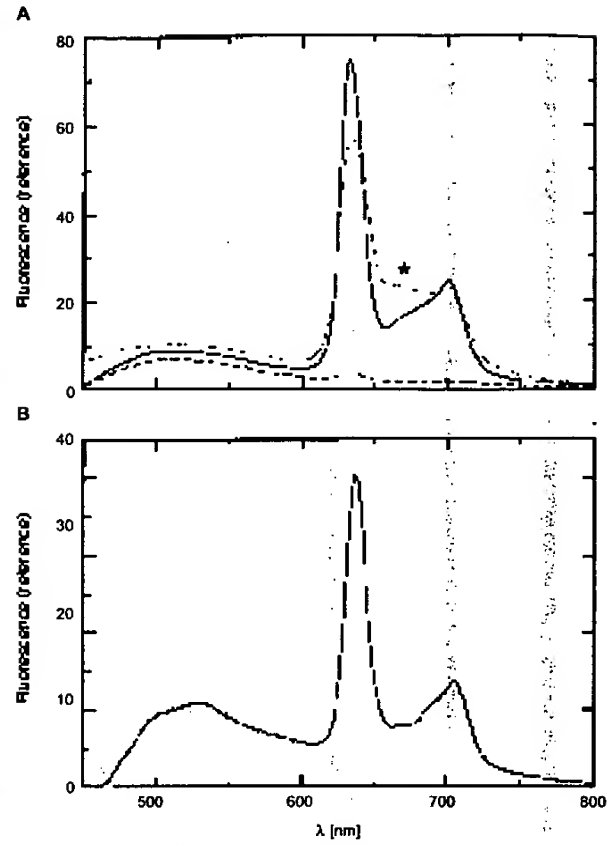

Figure 3 (A) Fluorescence spectra of h-ALA-induced PpIX (λ_{ex} = 405 nm) in normal mucosa (– – – –) and a papillary tumour (pTa G2, ——) after 4 h of instillation with 8 mM h-ALA (. . . .): a fluorescence peak (*) at 670 nm becomes visible due to photobleaching of PpIX. (B) Fluorescence spectra of ALA-induced PpIX (λ_{ex} = 405 nm) in a papillary tumour (pTa G2, ——) after 6 h of installation with 180 mM ALA)

Figure 2 demonstrates the advantageous use of h-ALA- induced PpIX for the fluorescence diagnosis of human bladder cancer. The two pictures show a sequence of a white light (Figure 2A) and a violet light (Figure 2B) examination after instillation of 8 mM of h-ALA over a period of 2 h (patient no. 10). White light illumination shows two papillary tumours (pTa G2) situated below the air bubble of the bladder under investigation. Fluorescence PD of the same area (Figure 2B) indicates a further lesion [flat papillary tumour (pTa G2)] which is barely detectable under white light.

Fluorescence findings and histopathological diagnosis

A total of 109 biopsies were taken under light-induced fluorescence from patients after instillation with h-ALA solutions. The correlation between the fluorescence findings and the histopathological analysis is summarized in Table 2. Thirty-two tissue samples were excised from healthy areas of the bladders investigated, containing eight samples, which were considered to be fluorescent. Histopathological diagnosis of the latter samples indicates the reasons for these 'false positive' responses. All these specimens showed tissular structures known for a higher cellular turnover, e.g. metaplasias, hyperplasias, chronic inflammation, or scar formation. In total, only six of the 77 biopsies taken from malignant and premalignant sites were not fluorescing. Three of these 'false negative' responses can be explained by nonoptimized conditions with regard to the concentrations of h-ALA applied (two moderate dysplasias; patient no. 4) as well as non-optimal incubation times (CIS; patient no. 1). A further CIS was missed (patient no. 13) probably due to an unusually long period of white light illumination preceding photodetection, resulting in the photobleaching of PpIX. Without exception, histopathologically-staged pta G1 or higher samples were found by fluorescence photodetection.

Fluorescence spectroscopy

Fluorescence emission spectra were measured on a total number of 24 patients (20 h-ALA, four ALA) (Table 1). ALA- and h-ALA-induced porphyrins were excited at 405 nm on both healthy and malignant areas in the human bladder. The emission from the urothelial surface was spectrally resolved between 450 and 800 nm. A comparison of the emission spectra after ALA and h-ALA exposure is plotted in Figure 3. The total fluorescence intensity is normalized to the reference. As shown in this Figure, the characteristic emission bands of PpIX at λ = 635 nm and λ = 708 nm after excitation in the Soret Band are clearly visible. According to the spectral shape, the fluorescence is attributed to PpIX. In none of the spectra recorded in vivo, an indication of porphyrins other than PpIX could be found. Depending on the duration of white and violet light examinations before fluorescence

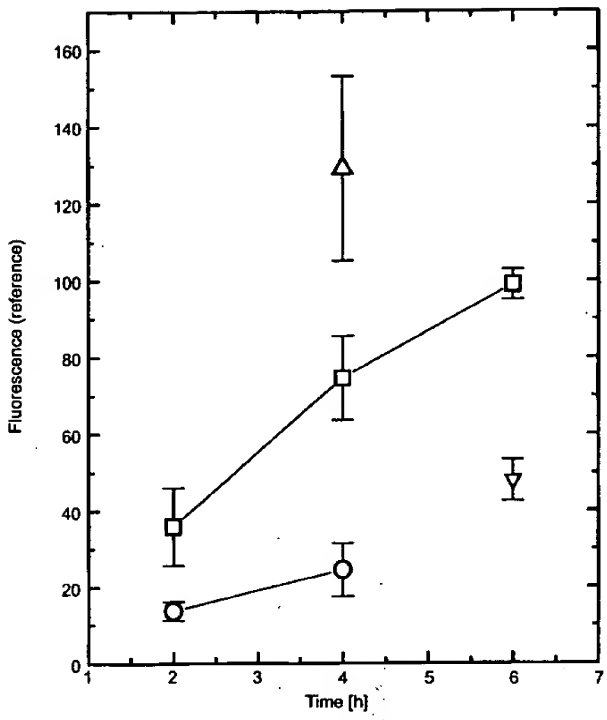

Figure 4 Effect of instillation time on the relative PpIX fluorescence intensity at 636 nm in papillary tumours (pTa G2) (□: 8 mM h-ALA; ○: 4 mM h-ALA; △: 8 mM h-ALA (2 h of instillation + 2 h of resting time); ▽: 180 mM ALA)

measurements, a peak, attributed to a PpIX photobleaching product, of around 665–675 nm appeared (dotted line in Figure 3A). The appearance of this supplementary fluorescence peak results in a line broadening because of overlapping fluorescence emission peaks and may suggest some degree of heterogeneity. In addition to the fluorescence emission spectra recorded on a papillary tumour pTa G2 after 4 h of h-ALA exposure, the corresponding spectra obtained on a healthy area were plotted (dashed line in Figure 3A). From these spectra, it can be seen that the healthy mucosa's autofluorescence of around 513 nm exceeds the two typical fluorescence peaks of PpIX at 636 nm and 708 nm. All samples taken from sites with these fluorescence characteristics were confirmed as healthy after histopathological examination. Evaluation of all fluorescence data available reveals that papillary tumour had the highest emission intensities. Premalignant lesions, such as dysplasias and carcinoma in situ, generally showed lower PpIX fluorescence intensities compared to malignant lesions. However, no direct relationship has been discovered between histopathological grading and relative fluorescence values.

Effect of exposure time and concentration

Three different solutions containing 50 mg (4 mM, four patients), 100 mg (8 mM, 15 patients) and 200 mg (16 mM, two patients) of h-ALA in 50 ml of the solvent were instilled in human bladders between 2 and 6 h prior to the fluorescence measurements (Table 1). Increased red fluorescence due to enhanced PpIX formation in pre-malignant and malignant lesions compared to the surrounding healthy sites was observed at all applied conditions. In order to quantify the resulting PpIX fluorescence, emission spectra were collected from different sites of the treated bladders. The fluorescence intensities of papillary tumours pTa graded G2 or G3 at 636 nm were chosen as standard in order to determine the influence of the different treatment conditions applied. This selection is based on the presence of this type of lesion in each bladder examined. Table 1 summarizes the influence of the different conditions on the relative fluorescence intensities of the PpIX emission band at 636 nm.

Analysis of the data available from patients exposed for 2 h (patient nos 1, 2, 5, 6, 7, 20 and 21) to different h-ALA concentrations indicates a strong concentration dependent on the PpIX fluorescence. It appears that, within 2 h, a solution of 8 mM h-ALA generates the highest fluorescence levels as compared to 4 mM and 16 mM of h-ALA. In Figure 4, the time course of the relative fluorescence intensity is plotted for h-ALA concentrations of 4 mM and 8 mM. An increase of fluorescence intensity with instillation time was observed in the two solutions. In addition, Figure 4 shows that, taking both the total fluorescence and the slope of the graphs into consideration, an instillation of 8 mM h-ALA solution is more efficient than that of a 4 mM solution.

In the course of our preliminary clinical study, a total of four patients (nos 8–11) were instilled under slightly different conditions. The patients' bladders were exposed to the solutions for 2 h. Following this exposure time, the bladders were emptied and the patients were allowed a supplementary resting time of 2 h. From Figure 4, it is clear that following this '(2+2)-concept' significantly enhanced fluorescence levels can be obtained compared to permanent exposure to the drug for 4 h.

The comparison of the relative fluorescence intensities of an 8 mM h-ALA solution and a 180 mM solution of ALA under similar conditions (4 h of instillation, 2 h of supplementary resting time) clearly demonstrates the advantage of using h-ALA. A treatment under these conditions with a topical 8 mM h-ALA solution resulted in a twofold increase of the fluorescence signal as compared to topical 180 mM ALA. After only 4 h of h-ALA exposure (8 mM), the relative fluorescence intensity already exceeded that induced by ALA (180 mM) 6 h after instillation.

DISCUSSION

Bladder cancer is a fairly common disease, appearing between the ages of 50 and 70 (Richie et al, 1989; Levi, 1993). This cancer, characterized by a high incidence (Levi, 1993), can appear in many distinct morphological forms, single or multiple, visible such as papillary or invisible such as 'flat' atypical lesions, mainly represented by low- or high-grade dysplasia or CIS. Bladder tumour multiplicity and the presence of these different forms of atypia are indicators of poor disease prognosis. Recognition of all visible or invisible lesions is therefore a prerequisite for any kind of treatment, with the aim of reducing the risk of progression or the rate of recurrence.

Although topical application of ALA has proved to be a helpful and reliable tool in fluorescence photodetection of invisible lesions in human bladder disease (Kriegmair et al, 1994; Jichlinski et al, 1996), some problems remain due to ALA's poor bioavailability. A small hydrophilic amino acid like ALA does not penetrate into all tissue compartments with great ease. Hence its concentration in tissue may remain relatively low and its distribution somewhat heterogeneous. Consequently, high drug doses over long instillation periods have to be used.

Three different concepts have been proposed to enhance the ALA-induced PpIX formation in deeper layers of the target tissue. Two of them are based on the use of chemicals, given along with ALA, in order to enhance both its penetration into deeper tissue layers and/or the total PpIX accumulation. This transepithelial penetration enhancement can be achieved either by prior dimethyl sulphoxide exposure of the targeted area (Peng et al, 1995) or by encapsulation of ALA into liposomes (Fukuda et al, 1992). The second approach uses agents interfering directly with the biosynthetic pathway of haem. Tetrapyrrol modulators, such as 1,10-phenanthroline (Rebeiz et al, 1996) and allyl-isopropyl-acetamide (AIA) (Schoenfeld et al, 1994) stimulating the enzymatic activity associated with PpIX formation. Iron chelators (e.g. ethylene-diaminotetraacetic acid (EDTA) (Hanania and Malik, 1992; Orenstein et al, 1995; Warloe et al, 1995), desferrioxamine (DFO) (Ortel et al, 1993) CP94 (Chang et al, 1997) have been shown to increase PpIX concentration by preventing the ferrochelatase-mediated insertion of iron into the tetrapyrrol ring. This study followed a third approach, based on the thesis that the transformation of the hydrophilic ALA into more lipophilic prodrugs will enhance drug uptake.

In view of the results obtained using esters of ALA in vitro (Kloek et al, 1996; Gaullier et al, 1997; Marti et al, 1999; Tyrrell et al, 1993) and in vivo (Kloek et al, 1996; Peng et al, 1996), it appeared reasonable to envisage developing such a substance for clinical tests in which superficial bladder carcinoma is detected by fluorescence and possibly even treated by PDT. From the variety of derivatives recently tested in our laboratory (Marti et al, 1999), we selected h-ALA as it represents a good compromise between water–urine solubility and lipophilicity. It also gave an excellent in-vitro dose drug response compared to ALA solutions. Furthermore, it can be synthesized with a fairly high yield and low cost. The goal of this first clinical study with h-ALA in urology was to evaluate the feasibility of fluorescence photodetection with this new agent and the advantages achieved by instillation of h-ALA as compared to ALA for use in the human bladder.

One criterion for the use of h-ALA as a potential candidate in replacing ALA, is the preservation of the outstanding selectivity of ALA-induced PpIX for malignant and pre-malignant tissues. Confirmed by histopathological examination, we have demonstrated that the fluorescence of PpIX in the urothelium induced by intravesically administered h-ALA correlated significantly with neoplastic lesions and was suitable for the detection of papillary tumours as well as for dysplasia and carcinoma in situ. The 7% rate of false negative responses found in the present study is comparable to the value given by Jichlinski et al in 1997 and slightly higher than that presented by the Munich group (Kriegmair et al, 1996). A total number of 28 biopsies were taken from areas proven to be benign. Only five of these samples revealed an enhanced red fluorescence under violet light irradiation, yielding a rate of falsely positive fluorescence findings of 17%. This result seems to be quite small compared to both the results of Kriegmair et al (1996) and Jichlinski et al (1997). But it may be explained by the small number of biopsies taken, or the fact that the fluorescence induced by the long-chain esters was found to be limited to the site of application (Peng et al, 1996), hence no supplementary PpIX build-up from systemic ALA uptake is observed.

Clinical fluorescence spectroscopy has been used for measuring the PpIX accumulation kinetics, indicating an increase of h-ALA-induced PpIX with time in the human bladder within 6 h. A quantitative comparison of the fluorescence intensities at 636 nm following similar instillation conditions with solutions of 180 mM of ALA or 8 mM of h-ALA, respectively, clearly shows the advantages of h-ALA-induced PpIX. The more than twofold increase of the fluorescence signal due to the use of h-ALA is in good agreement with the in vivo results of Kloek et al (1996).

The time course of the PpIX fluorescence intensity in neoplastic tissues shows that, following 8 mM h-ALA exposure for 2 or 4 h, synthesis of PpIX continues within almost 2 h after termination of the instillation. In this time range, the fluorescence intensity increases 400% (2 h of exposure, 2 h of resting time) and 25% (4 h of exposure, 2 h of resting time) respectively.

The significant increase of the fluorescence signal using the '(2+2)-concept' as compared to a permanent exposure to drug for 4 h, as well as the strong dependence on the instilled h-ALA concentration, can be explained by an interference of high ALA concentrations with the biosynthetic pathway of haem. This observation was confirmed by in vitro experiments made by Gaullier et al (1997) and Marti et al (1999) with several ALA esters including h-ALA. Whereas the transport of ALA across the lipid bilayer of cell membranes probably represents a bottleneck in the PpIX formation, the enhanced uptake of lipophilic h-ALA may saturate the intracellular PpIX biosynthesis. This saturation might cause a negative feedback to enzymatic activity. Furthermore, high intracellular ALA concentrations have been shown to be cytotoxic. A high cellular ALA content may induce the release of Ca^{2+} from mitochondria, mitochondria swelling and uncouple respiration (Hermes-Lima, 1995). It can also cause ferritin iron release (Berg et al, 1996) or mediate the formation of 8-hydroxy-2′-deoxyguanosine in DNA (Fraga et al, 1994).

The results presented in this study have shown that a 2 h instillation of h-ALA (8 mM) provides sufficient PpIX fluorescence for reliable photodetection of malignant and pre-malignant lesions. This reduction in instillation time to only 2 h significantly increases the patient's comfort. Moreover, this makes outpatient treatment feasible and helps to cut costs in view of the excessively increasing cost of hospitalization. Finally, the reduction of the drug dose will decrease drug cost and the potential risk of mild complications provoked by ALA, recently reported by Rick et al (1997).

While for reliable fluorescence photodetection a 2 h instillation of 8 mM h-ALA has been shown to give satisfactory results, other conditions must be fulfilled with respect to an efficient bladder cancer therapy by PDT. Among other factors, the two key parameters of high concentration of the photosensitizer and its homogeneous distribution in the target tissue play a major role for the effectiveness of PDT.

Fluorescence microscopic studies showed that, after topical application of ALA, the PpIX was restricted to the superficial layers of the bladder tumours (Steinbach et al, 1994). On the contrary, preliminary fluorescence microscopic studies on some biopsies, taken in this study (data not shown), as well as the in vitro studies of Marti et al (1999) demonstrated homogeneously distributed PpIX fluorescence over the entire urothelium after topical application of h-ALA solutions.

Considering the photobleaching of porphyrins during irradiation (Rotomski et al, 1996; Bezdetnaya et al, 1996; Moan et al, 1997), a threshold concentration of PpIX necessary for tissue destruction, and the high selectivity of h-ALA-induced PpIX, a small PpIX amount in healthy areas of the bladder, observed in this study will

probably not induce any damage in these regions. However, the twofold increase of PpIX fluorescence after 6 h in neoplastic tissues by using h-ALA may further enhance the PDT effect as compared to the use of ALA. The latter appeared insufficient as observed in recent studies (Kriegmair et al, 1996).

It can be concluded that the use of h-ALA is a promising way to improve the photodetection of neoplastic and pre-neoplastic lesions as compared to ALA. In future, h-ALA may replace the use of ALA for clinical intravesical instillation because it is easy to use, real time observation without major auxiliary devices is possible and it is relatively cheap. Finally, it looks more promising as a PDT agent than ALA itself.

ACKNOWLEDGEMENTS

The authors are grateful to the Swiss 'Fonds National', the Common Research Program in biomedical technology between Lausanne Hospital (CHUV), the Swiss Federal Institute of Technology (EPFL), Lausanne University (UNIL), the Swiss National Priority Program in Optics, and the 'Fonds Vaud-Geneva' for their financial support. Norbert Lange thanks Patrick Gerber (ICO, University of Lausanne) for many fruitful discussions. The Deutsche Forschungsgemeinschaft (DFG), Bonn, Germany, provided the grant for Dr N Lange.

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CLINICAL SPECTROSCOPY MEASUREMENT
PRIX AS COMPARED TO ALA
instillation + 2 hours resting time

fluorescence [r. u.]

80
60
40
20
0

500 600 700 800

λ [nm]

180 mM ALA
8 mM h-ALA

TUMOR STAGING:
pTa G2

ELSEVIER

Journal of Photochemistry and Photobiology B: Biology 34 (1996) 95–96

Journal of PHOTOCHEMISTRY AND PHOTOBIOLOGY B: BIOLOGY

News and Views

Build-up of esterified aminolevulinic-acid-derivative-induced porphyrin fluorescence in normal mouse skin

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During the past 5 years, aminolevulinic-acid(ALA)-mediated photodynamic therapy (PDT) of cancer is one of the most rapidly developing fields in PDT research. Since Kennedy et al. [1] proposed the use of topically ALA-based PDT of cutaneous diseases in 1990, much interest has arisen in many laboratories [2–4]. In particular, promising clinical results have been obtained in the treatment of superficial basal cell carcinoma [5,6]. However, this modality is not optimally effective to sensitize the nodular lesions to complete destruction, probably owing to the limited skin penetration of ALA and production of ALA-induced porphyrins in the deep layers of the lesions [7]. Thus enhancement of both ALA absorption and ALA-derived porphyrin production in the nodular lesions is a crucial factor to improve the technique. Recently we have studied the effects of methylester, ethylester and propylester derivatives of ALA (H_2N–CH_2COCH_2–CH_2COO–R; R can be CH_3, CH_2–CH_3 or CH_2–CH_2–CH_3) on production of porphyrins in the normal skin of female Balb/c athymic nude mice. We found, by means of an optical-fiber-based point monitoring system in situ, that a slight porphyrin fluorescence was built up already 1 h after topical application of the derivatives (20% in a cream) in the right flank of the mice. The maximum fluorescence intensity was found 14 h after the application for both free ALA and its ester derivatives, but the porphyrin fluorescence induced by the ALA esters in the skin was stronger than that induced by free ALA (Fig. 1). Moreover, as can be seen in Fig. 2, 14 h after the topical application no fluorescence of ALA-ester-induced porphyrins was detected in other areas than that in which the cream was applied (ear, liver, muscle and brain). However, in the case of free ALA, a significant fluorescence was seen in the skin outside the area of application. Fluorescence imaging of the skin treated with the three derivatives showed fluorescence of the ester derivative-induced porphyrins in the epidermis, epithelial hair follicles and sebaceous glands. In the case of intraperitoneal (i.p.) injection (150 mg

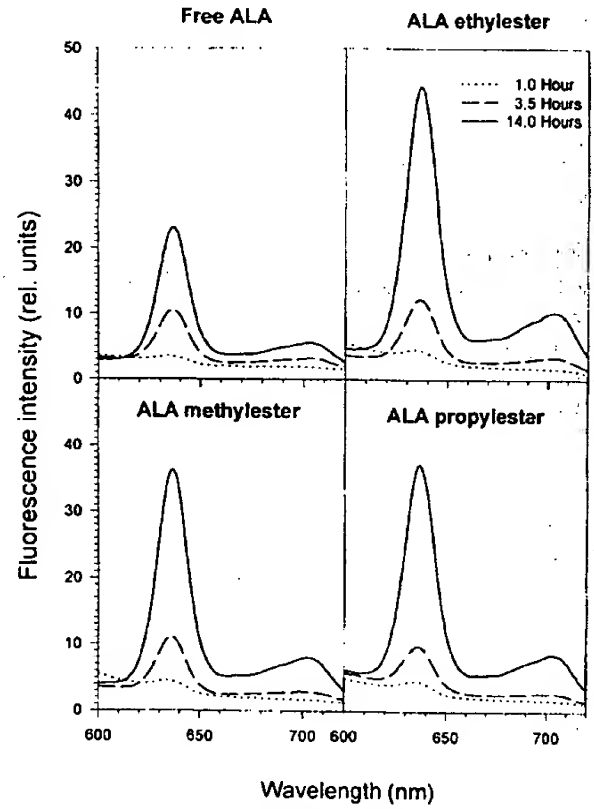

Fig. 1. Fluorescence spectra of free ALA- and its three ester derivative-induced porphyrins in the normal mouse skin in situ at various time intervals (as indicated) after topical application.

kg^{-1}) the fluorescence of the ALA methylester-induced porphyrins in the skin was built up 15 min after injection. The peak value was found at around 1–2 h and disappeared within 12 h post injection. This kinetic pattern was similar to that of the fluorescence of free ALA-induced porphyrins in the skin

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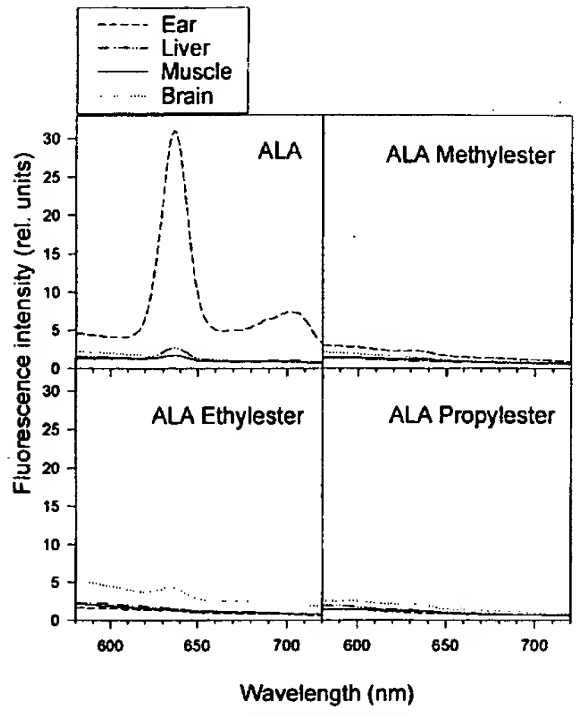

Fig. 2. Fluorescence spectra of free ALA- and its three ester derivative-induced porphyrins in the ear, liver, muscle and brain of the mice 14 h after topical application.

following i.p. injection of the same dose, although the fluorescence decreased more rapidly in the case of the ester than in the case of free ALA. The present data indicate that all derivatives studied were taken up, de-esterified and finally converted into porphyrins in the epidermis, epithelial hair follicles and sebaceous glands of the nude mice with a higher porphyrin production than that of free ALA. This is in agreement with our preliminary results obtained in a study of human nodular basal cell carcinoma that demonstrated that the fluorescence of the ALA ester-induced porphyrins was built up more rapidly with a higher intensity and a more homogenous distribution than those of free ALA-induced porphyrins in the lesions [8]. Interestingly, a strong fluorescence of free ALA-induced porphyrins was found in regions of the skin outside the area where the cream was topically applied. This indicates that, after topical application, free ALA is transported in the blood and porphyrins may subsequently be formed in all organs containing the enzymes of the heme synthesis pathway or ALA-induced porphyrins are initially formed in the liver and then transported to other tissues via blood circulation. This may lead to skin photosensitivity in areas where free ALA is even not topically applied. However, none of the ester derivatives studied induced porphyrin fluorescence at distant skin sites.

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